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# Study of the immunopathogenesis of narcolepsy type-1 using original mouse models

David Frieser

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# THÈSE

**En vue de l'obtention du  
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**

**Délivré par l'Université Toulouse 3 - Paul Sabatier**

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Présentée et soutenue par :

**David FRIESER**

Le 19 décembre 2022

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**Analyse de l'immunopathogénèse de la narcolepsie de type 1  
à l'aide des modèles animaux**

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Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

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**INFINITY - Institut Toulousain des Maladies  
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Thèse dirigée par :

**Roland LIBLAU et Frédéric MASSON**

---

## Jury

**M. Joost SMOLDERS,**

Examineur

**M. Joost P.M. van Meerwijk**

Examineur

**Mme Katia BONIFACE,**

Rapporteuse

**M. Romain MARIGNIER,**

Rapporteur

**M. Martin KERSCHENSTEINER,**

Examineur

**M. Roland LIBLAU,**

Directeur de thèse

**M. Frédéric MASSON,**

Co-directeur de thèse



Université  
de Toulouse

# Ph.D. Thesis

**Specialty:** Immunology

**Specialization:** autoimmune diseases of the central nervous system

**Title**

" Study of the immunopathogenesis of narcolepsy type-1  
using original mouse models "

**Written at**

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Inserm UMR1291 - INFINITY (Team 4)

CHU Purpan | 31024 Toulouse | France

**Written by**

David Frieser (M. Sc.)

**Under the supervision of**

Prof. Dr. Roland Liblau

Dr. Frederic Masson

**Date:** 29<sup>th</sup> of November, 2022

**Jury**

**M. Joost SMOLDERS,**

**M. Joost P.M. van Meerwijk**

**Mme Katia BONIFACE,**

**M. Romain MARIGNIER,**

**M. Martin KERSCHENSTEINER,**

**M. Roland LIBLAU,**

**M. Frédéric MASSON,**

Examineur

Examineur

Rapporteuse

Rapporteur

Examineur

Directeur de thèse

Co-directeur de thèse

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<sup>1</sup> All Figures were created on Biorencor.com<sup>®</sup> if not otherwise specified

## Abbreviations

<b>AD</b>	Alzheimer disease
<b>APC</b>	Antigen-presenting cell
<b>Cas9</b>	CRISPR-associated protein 9
<b>CCL2</b>	Chemokine (C-C motif) ligand 2
<b>CD69</b>	Cluster of Differentiation 69
<b>CNS</b>	Central nervous system
<b>CRISPR</b>	clustered regularly interspaced short palindromic repeats/
<b>cTEC</b>	cortical thymic epithelial cell
<b>CTL</b>	cytotoxic T lymphocyte
<b>CXCR3</b>	CXC chemokine receptor 3
<b>CXCL9</b>	Chemokine (C-X-C motif) ligand 9
<b>DC</b>	Dendritic cell
<b>DNA</b>	Desoxy ribonucleic acid
<b>GM-CSF</b>	Granulocyte-macrophage colony-stimulating factor
<b>Grz B</b>	Granzyme B
<b>IL-2</b>	Interleukin-2
<b>IFN<math>\gamma</math></b>	Interferon $\gamma$
<b>IFR</b>	Interfollicular region
<b>IgG</b>	Immunoglobulin G
<b>JCV</b>	John Cunningham virus
<b>JEV</b>	Japanese encephalitis virus
<b>LCMV</b>	Lymphocytic choriomeningitis virus
<b>LFA1</b>	Lymphocyte function-associated antigen 1 (CD11a)
<b>LN</b>	Lymphnode
<b>mAb</b>	monoclonal Antibody
<b>MHC</b>	Major histocompatibility complex
<b>MS</b>	Multiple sclerosis
<b>mTEC</b>	medullary thymic epithelial cell
<b>PBMC</b>	Peripheral blood myeloid cell
<b>PD</b>	Parkinson disease
<b>PD-1</b>	Programmed cell death protein 1
<b>RE</b>	Rasmussen encephalitis
<b>RNA</b>	Ribonucleic acid
<b>mRNA</b>	messenger RNA
<b>scRNAseq</b>	single cell RNA sequencing
<b>shRNA</b>	short hairpin RNA
<b>SLO</b>	secondary lymphoid tissues
<b>TCR</b>	T-cell receptor
<b>T<sub>CM</sub></b>	T central memory
<b>T<sub>EM</sub></b>	T effector memory
<b>T<sub>FH</sub></b>	T follicular helper
<b>T<sub>RM</sub></b>	T resident memory
<b>Treg</b>	T regulatory
<b>TF</b>	Transcription factor
<b>Tg</b>	<i>Toxoplasma gondii</i>
<b>TGF<math>\beta</math></b>	Transforming growth factor $\beta$
<b>TNF</b>	Tumor necrosis factor
<b>VLA4</b>	Integrin $\alpha4\beta1$ (very late antigen-4)
<b>WNV</b>	West Nile Virus

## Abstract

Narcolepsy type 1 (NT1) is the most severe cause of hypersomnia in humans with a unique disease course. With a global prevalence of 200-500 per million individuals, it represents a rare disease. NT1 usually begins between the ages of 10 and 20 years with the sudden onset of persistent daytime sleepiness and night-time sleep disruption. Another characteristic of NT1 is cataplexy — sudden episodes of partial or complete paralysis of voluntary muscles triggered by strong, mostly positive emotions such as laughing.

The mechanism underlying NT1 is the deficiency of Orexin-(or Hypocretin) signaling as this hormone is drastically reduced in post-mortem brains of patients and their cerebral spinal fluid (CSF). The loss of these ~70.000 Orexin-neurons, located in the human tuberal hypothalamus, emerges later in life and is closely linked to the onset of symptoms. Nevertheless, the reason for the sudden loss of these neurons remains elusive.

More than 98% of people with NT1 carry the HLA-DQB1\*06:02 allele (providing an odds ratio of >250), a finding that makes the strongest association between an HLA allele and disease known. This led to the autoimmune hypothesis for the development of NT1. In recent studies, autoreactive CD4 and CD8 T cells with reactivity towards Orexin have been identified in blood samples from patients with NT1, which further support the auto-immune origin of NT1.

In August 2010 reports of a possible association between exposure to the Pandemrix<sup>®</sup> vaccine against the H1N1 influenza virus and the occurrence of narcolepsy in children and adolescents attracted high media attention. Data from several retrospective studies in European countries showed 3- to 14-fold increase in the relative incidence of narcolepsy in vaccinated individuals, but the mechanisms underlying such association are still unclear.

Our team demonstrated that the adoptive transfer of autoreactive CD8<sup>+</sup> T cells against Orexin-neurons in mice caused the development of sleep fragmentation and cataplexy episodes, thus providing an autoimmune model for NT1. Building on this publication, this Ph.D. thesis aimed to study the interaction of autoreactive T cells with orexin neurons.

Furthermore, a novel mouse model was developed, induced by Pandemrix<sup>®</sup> vaccination to study possible mechanisms by which the vaccine could have induced NT1 onset. The in-depth characterization of the autoreactive T cells in this model led us to the discovery of a tissue-resident phenotype of the CD8 T cells in the hypothalamus, that might be essential to induce the progressive loss of orexin neurons.



We used high-dimensional flow-cytometry, histopathology, and RNA sequencing data to study the phenotype and heterogeneity of tissue-resident CD8 T cells and their role in progressive neuronal degradation. The chronic inflamed state of the hypothalamus directly correlated with the number of tissue-resident CD8 T cells in the hypothalamus. We were able to partially rescue the neuronal loss by the depletion of tissue-resident CD8 T cells once we targeted a resident follicular CD4 T helper population that seems to be essential for the maintenance of the CD8 tissue residency program.

Finally, we identified IFN $\gamma$  to be a crucial cytokine in the maintenance of tissue-resident CD8 T cells. IFN $\gamma$  seems to install an autocrine signaling loop in hypothalamic tissue-resident CD8 T cells, that helps them to survive in the central nervous system (CNS). Once we inhibited this signaling loop, CD8 T cells were unable to persist in the hypothalamus. The results in this autoimmune model for NT1 might serve as a future therapeutic strategies for other CNS autoimmune diseases.

## Sommaire

La narcolepsie de type 1 (NT1) est la cause la plus grave d'hypersomnie chez l'Homme. Avec une prévalence mondiale de 1 cas pour 2 000 individus, elle représente une maladie rare. La NT1 débute généralement entre 10 et 20 ans par l'apparition soudaine d'une somnolence diurne persistante et d'une perturbation du sommeil nocturne. Une autre caractéristique de la NT1 est la cataplexie - des épisodes soudains de paralysie partielle des muscles volontaires déclenchés par des émotions fortes.

Le mécanisme qui sous-tend la NT1 est la déficience de la signalisation de l'orexine, car cette hormone est considérablement réduite dans le cerveau post-mortem des patients et dans leur liquide céphalo-rachidien. La perte de ces ~70'000 neurones à orexine, situés dans l'hypothalamus tubéral humain, se manifeste plus tard dans la vie et est étroitement liée à l'apparition des symptômes. Néanmoins, la raison de la perte soudaine de ces neurones reste inconnue.

Plus de 98 % des personnes atteintes de la NT1 sont porteuses de l'allèle HLA-DQB1\*06:02 (ce qui donne un ratio OD >250), ce qui constitue la plus forte association connue entre un allèle HLA et une maladie. Cela a conduit à l'hypothèse d'une cause auto-immune sous-tendant le développement de la NT1. Dans des études récentes, des lymphocytes T CD4 et T CD8 autoréactives présentant une réactivité envers l'orexine ont été identifiées dans des échantillons de sang de patients atteints de NT1, renforçant l'hypothèse auto-immune de la NT1.

En août 2010, les rapports sur une association possible entre l'exposition au vaccin Pandemrix® contre le virus de la grippe H1N1 et l'apparition de la narcolepsie chez les enfants et les adolescents ont retenu l'attention des médias. Les données de plusieurs études rétrospectives menées dans des pays européens ont montré une augmentation de 3 à 14 fois de l'incidence relative de la narcolepsie chez les enfants vaccinées, mais les mécanismes qui sous-tendent cette association ne sont toujours pas clairs.

Notre équipe a démontré que le transfert adoptif de cellules T CD8 autoréactives contre les neurones à orexine chez la souris induit une perte de neurones à orexine associée à une fragmentation du sommeil et à des épisodes de cataplexie, fournissant ainsi un modèle auto-immun de NT1. Sur la base de cette publication, ma thèse visait à étudier l'interaction des lymphocytes T autoréactifs avec les neurones à orexine.

En outre, un nouveau modèle de souris a été développé, induit par la vaccination avec le vaccin antigrippale Pandemrix<sup>®</sup>, afin d'étudier les mécanismes possibles par lesquels le vaccin aurait pu induire l'apparition de la NT1. La caractérisation approfondie des cellules T autoréactives dans ce modèle nous a conduit à la découverte d'un phénotype de lymphocytes T CD8 résidant dans l'hypothalamus, qui pourrait être essentiel pour induire la mort des neurones à orexine progressives.

Nous avons utilisé des données de cytométrie en flux, d'histopathologie et de séquençage de l'ARN sur cellules uniques pour étudier le phénotype et l'hétérogénéité des lymphocytes T CD8 résidant dans les tissus et leur rôle dans la perte progressive des neurones. L'état d'inflammation chronique de l'hypothalamus était directement corrélé au nombre de lymphocytes T CD8 résidant dans l'hypothalamus. La perte neuronale était compensée par la déplétion des lymphocytes T CD8 résidant dans le tissu par la déplétion d'une population de T CD4 folliculaires résidants. Cette population semble être essentielle pour le maintien du programme de résidence des CD8 dans le tissu.

Enfin, nous avons identifié l'IFN $\gamma$  comme une cytokine importante pour le maintien des cellules T CD8 résidentes des tissus. L'IFN $\gamma$  semble installer une boucle de signalisation autocrine dans les cellules T CD8 résidentes dans le système nerveuse central (SNC). Une fois que nous avons inhibé cette boucle de signalisation, les lymphocytes T CD8 étaient incapables de persister dans le SNC. Nos résultats dans ce modèle auto-immun pour le NT peuvent ouvrir le chemin pour le développement de nouvelles thérapeutiques pour d'autres maladies auto-immunes du SNC.

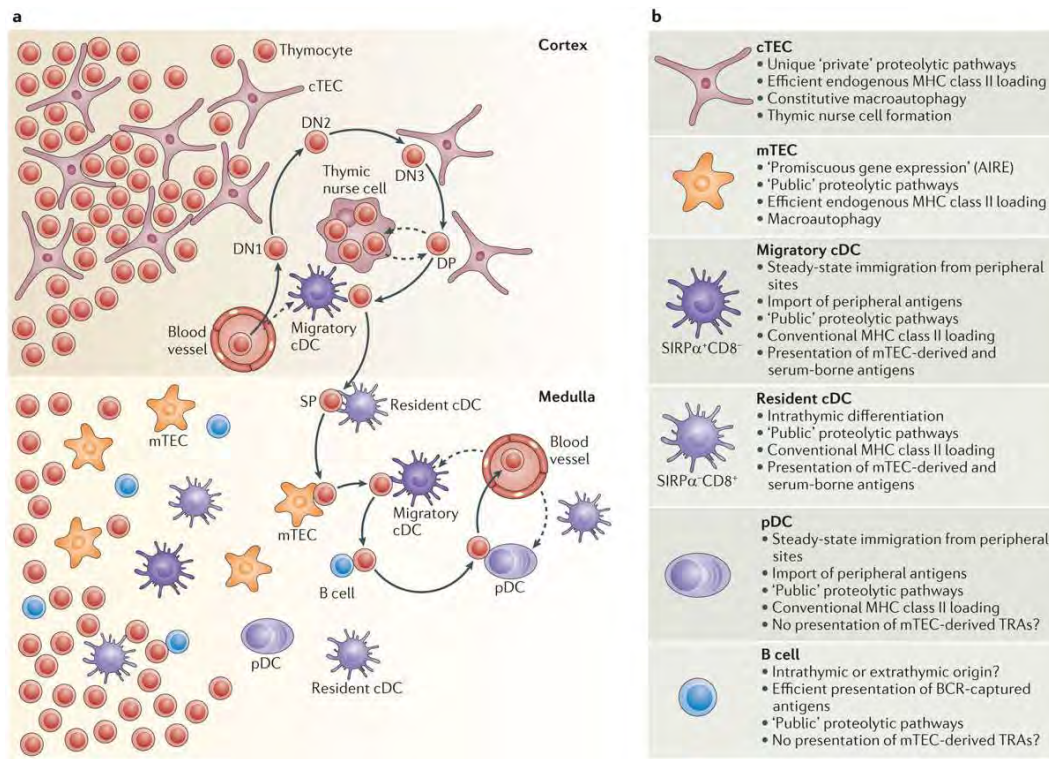
# 1 Introduction

## 1.1 T cell activation and differentiation

### 1.1.1 Spatial and transcriptional aspects of T cell differentiation

Thymocytes arise from a hematopoietic stem cell precursor. They are generated in the thymus during their development, wherein during differentiation, functional CD4 or CD8 T cells are selected (Germain et al. 2002; **Fig. 1.1**). In the cortex, cortical thymic epithelial cells (cTECs) form so-called nurse cells to positively select T cells that bind to the generated MHC:peptide complexes with low affinity (Nakagawa et al. 2012). The Thymoproteasome subunit- $\beta$ 5T processes antigens that are presented via the Major histocompatibility complex I (MHC-I) to CD8 T cells possessing functional T cell receptors (TCR; Florea et al. 2010). In the same way, the Thymus-specific serine proteases cleave antigens that are presented to CD4 T cells by the MHC-II complex (Gommeaux et al. 2009). All T cells with non-binding TCRs are eliminated - this is why this process is termed positive selection.

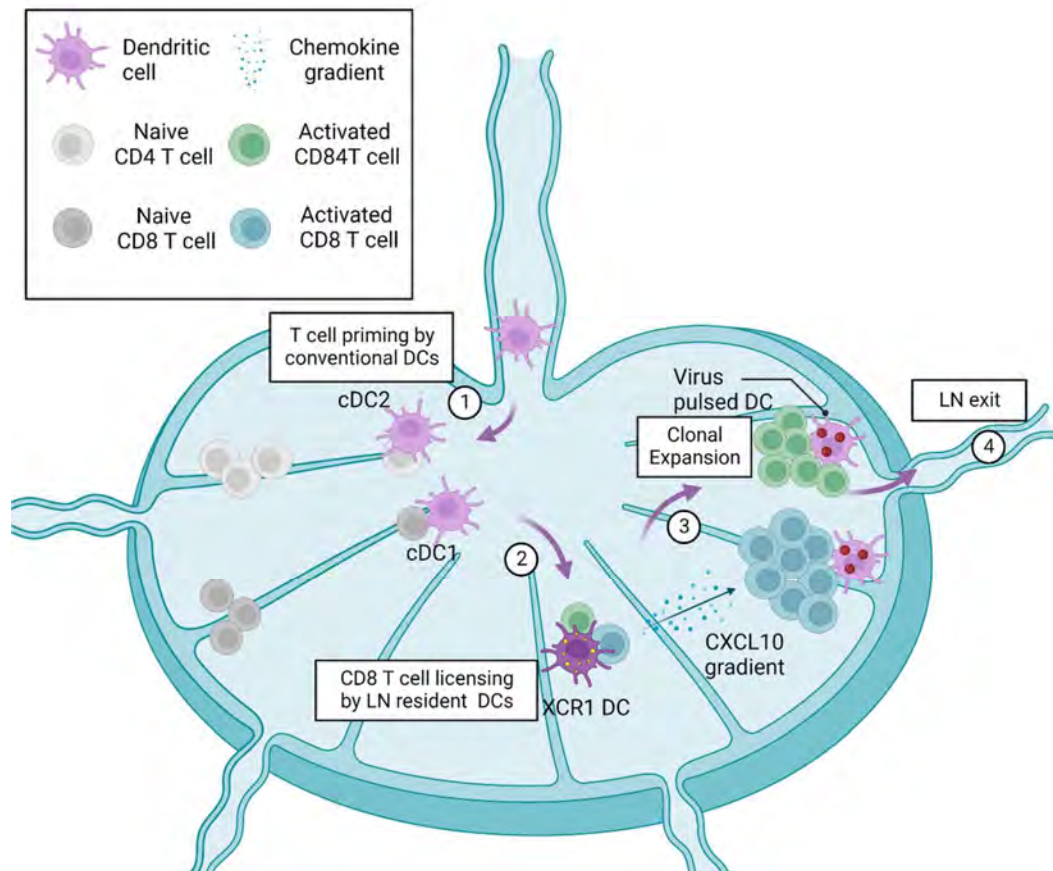
The negative selection takes place in the medulla, where T cells encounter another type of antigen-presenting cells (APCs): The medullary thymic epithelial cells (mTECs). These can present to T cells antigens from the host tissue, which is regulated in part by the transcription factor AIRE. This mechanism ensures central tolerance, avoiding reaction of T cells against self-antigens (Mathis et al. 2009). This was brought to evidence as the genetic depletion of AIRE results in a variety of autoimmune diseases (Taniguchi et al. 2012). When a TCR binds with high affinity to self-antigens, the T cell undergoes apoptosis. However, once the TCR of a CD4 T cell binds to a self-antigen with intermediate affinity it induces the development of T regulatory (Treg) cells (Moran et al. 2011). Through this selection process, T cells are endowed with functional TCRs that recognize foreign but not self-antigens. Moreover, an additional layer of security is provided by the development of Treg cells (expressing mostly self-reactive TCR) in the thymus that can control in the periphery possibly arising autoreactive T cells. Nevertheless, T cells with low-affinity autoreactive TCRs are able to evade negative selection. For this reason, immunization with the MOG<sub>35-55</sub> peptide in mice results in the development of an autoimmune disease of the CNS. Furthermore, 4.5% of the world population are affected by more than 76 different autoimmune diseases and the number of autoimmune diseases is increasing (Chauhan et al. 2019).



**Figure 1.1 Spatial aspects of T cell development in the thymus. (A)** Successive stages of double-negative (DN) T cell development are accompanied by an outward movement of thymocytes towards the subcapsular zone. Subsequent T cells encounter cortical thymic epithelial cells (cTECs) for positively selecting ligands. At this stage, DP thymocytes may be engulfed by cTECs and form so-called thymic nurse cells. Positively selected CD4 or CD8 lineage-committed thymocytes relocate into the medulla by directed migration. Upon reaching the medulla, single-positive (SP) cells encounter medullary thymic epithelial cells (mTECs). Solid arrows indicate the main migratory pathways that are involved in thymocyte selection. Dashed arrows indicate other relevant migratory pathways. **(B)** Key functional properties of thymic APCs. AIRE, autoimmune regulator; BCR, B cell receptor; TRA, tissue-restricted antigen (Klein et al. 2014)

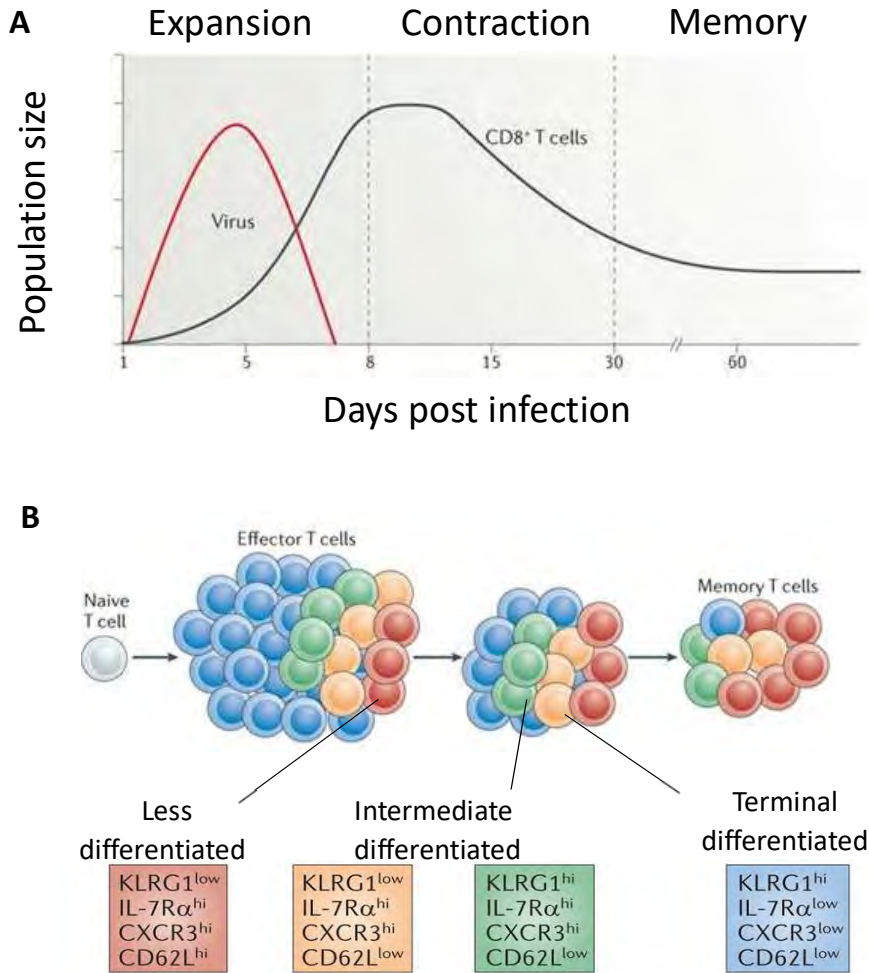
Naïve T cells, which have not encountered the antigen through their TCR, then exit the medulla to the blood circulation and home in secondary lymphoid tissues (SLOs), such as the lymph nodes (LNs), spleen and the Peyer’s Patches (Weninger et al. 2001). T cell activation takes place first in the interfollicular regions (IFRs) of LNs, where conventional dendritic cells (cDCs) encounter a naïve T cell with its cognate TCR and form cell clusters (Alexandre et al. 2018). In these steps, CD4 and CD8 T cells are primed by cDC2 (mainly CD4 T cells) and cDC1 (mainly CD8 T cells) subpopulations. T cell activation requires 24 to 48 hours of contact between the naïve T cell and an APC, during which the T cell increases in size (blasting), and on the epigenetic level chromatin-remodeling occurs enabling them, later on, to rapidly induce effector functions upon antigen re-encounter (Bevington et al. 2017). After the first priming step, T cells move further within the LN into the paracortical regions, where they encounter LN-resident XCR1<sup>+</sup> DCs.

CD4 T helper cells activate XCR1<sup>+</sup> DCs, which leads to cytokine production and upregulation of co-stimulatory molecules, which then serve as secondary priming signal for optimal CD8 T cell activation (Eickhoff et al. 2015; see **1.1.2** for more details). These movements within the LN are driven by chemokine gradients produced mainly by DCs. Upon viral infection, high production of CXCL10 by DCs in the paracortex drives CXCR3 expressing T cells to once again migrate out of the paracortex into IFRs (Duckworth et al. 2021; **Fig. 1.2**).



**Figure 1.2 Spatial aspects of T cell priming and differentiation.** After conventional DCs (cDCs) pulsed with their antigen enter the LN, CD4 T cells get activated by cDC2 and CD8 T cells by cDC1 in the IFR. This first priming step takes 24-48 hrs, after that these T cells migrate from the IFRs to the paracortex where special LN-resident XCR1<sup>+</sup> DCs confer the second priming step, which is also known as licensing of the CD8 T cells by CD4 T cell help. Once new DCs charged or infected with the cognate antigen (Virus) enter into the LN, they produce a CXCL10 gradient, which causes the T cells to migrate into IFRs again, where they undergo clonal expansion and exit the LN.

T cell differentiation can then be divided into three phases: i) clonal expansion ii) contraction and iii) memory phase (**Fig. 1.3A**). During clonal expansion, effector populations increase in size up to 1000-fold within one week (Pepper et al. 2011). In the contraction phase, less than 10% of those activated T cells survive, from which memory populations then arise (Marshall et al. 2011).



**Figure 1.3 Expansion, Contraction, and Memory phase during T cell development.** (A) After T cell priming and activation, naïve T cells undergo clonal expansion within one week. After the pathogen is cleared, many T cells die in the contraction phase and only 5-10% remain, which form the antigen-specific memory population. (B) A model shows that among the memory T cells very few cells express markers of terminal effector T cells (blue box), and more express markers of less differentiated cells (red box). This graphic should illustrate that these markers are not definitive, but also that some cells from the blue box phenotype differentiate into memory cells, *adopted from Kaech et al. 2012*

Given the large diversity of memory CD4 and CD8 T cells depending on the type of immune response, the most important transcription factors, those involved in CD8 T cell differentiation upon viral (such as LCMV) or bacterial (such as *Listeria monocytogenes*) infections, will be discussed, as the majority of studies made use of these pathogens.

Early surface markers such as IL-7R $\alpha$  and KLRG1 distinguish terminal effector T cells (T<sub>TE</sub>) that undergo apoptosis from memory T cell precursors (T<sub>MP</sub>) that undergo self-renewal, expressing IL-7R $\alpha$  and CD27 among others (Kaech et al. 2012). Among KLRG1<sup>hi</sup> IL-7R $\alpha$ <sup>low</sup> populations, most cells differentiate into terminally effector T cells with the highest cytotoxic potential, which subsequently die. In contrast, KLRG1<sup>low</sup> IL-7R $\alpha$ <sup>hi</sup> cells represent long-lived memory T cell subsets (**Fig. 1.3B**; Croom et al. 2011). This is controlled by a set of transcription factors.

IL-12 secreted by DCs during antigen-presentation drives the expression of the transcription factor (TF) T-Bet via mTOR signaling (Rao et al. 2010). T-Bet is important for the equipment of effector function, including cytokine production of Interferon- $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor (TNF). Further, CXCR3 and CXCR4 are expressed enabling their migration patterns out of the SLOs (Joshi et al. 2007). Furthermore, IL-12 together with IL-2 induce BLIMP-1 expression in CD8 T cells, which was shown to enhance cytotoxic functions such as Granzyme B and Perforin production (Kallies et al. 2009). Another TF, ID2, is induced, which plays a pivotal role in the survival of effector T cells in the naïve – effector transition, triggering the expression of pro-survival molecules such as Bcl2 and BIM (Yang et al. 2011, Masson et al. 2013, Knell et al. 2013). STAT4 is activated upon IL-12 and STAT1 upon IFN $\gamma$  signaling. Both TFs were shown to promote naïve – effector transition (Cui et al. 2011). They act by increasing Blimp1 and T-Bet; IL-2R and IL-12R levels, therefore, providing a positive feedback loop (Pipkin et al. 2010).

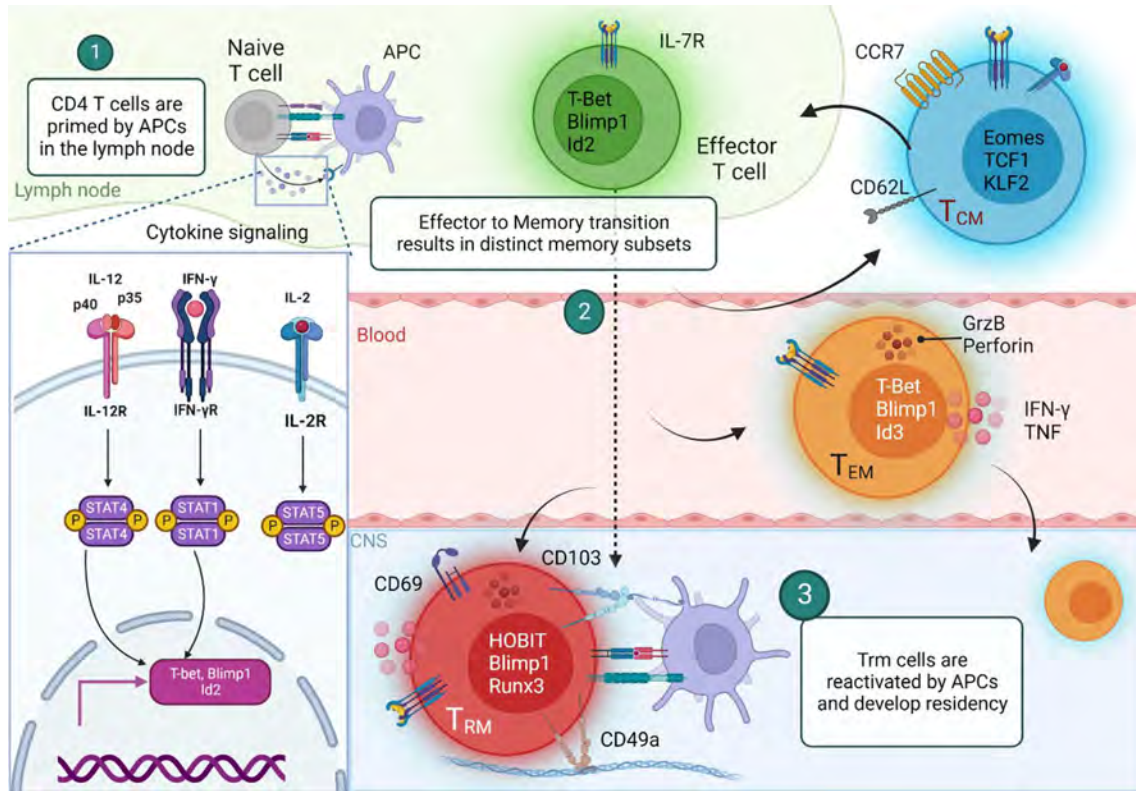
During the effector - memory transition, the balance of several transcription factors changes. IL-2, IL-12, and IL-21 additionally drive the expression of Eomes that also confers effector functions such as IFN $\gamma$ , Granzyme B, and Perforin production (Intlekofer et al. 2005). Furthermore, Eomes upregulates the expression of IL-7R $\alpha$  and IL-15R $\alpha$ , the former being important for homeostatic proliferation and the latter for the long-term survival of memory T cells (Banerjee et al. 2010; Xu et al. 2016). It was shown that increased Eomes expression results in lower T-Bet expression, which is important for efficient effector - memory transition (Joshi et al. 2011). STAT3 is as well induced upon IL-21 signaling and is crucial for the generation of memory T cells, shown both in mice and in humans (Cui et al. 2011, Siegel et al. 2011). STAT3 then downregulates STAT4 expression. In line with this interplay, ID3 is expressed at higher levels in the effector - memory transition and downregulates ID-2 expression (Ji et al. 2011). This provides evidence that the differentiation steps are regulated by the fine balance of TFs, that share overlapping functions but are expressed differently during T cell differentiation.

Several T cell memory subsets have been described depending on their migratory capacities (Sallusto et al. 1999), which are driven by specific TFs. Therefore, focusing on those memory subsets helps to narrow down subset-specific TFs. Central-memory CD8 and CD4 T cells (T<sub>CM</sub>) remain in the lymph nodes or lymphoid structures and express CD62L and CCR7, which are homing receptors for secondary lymphoid organs (SLOs). They express low levels of BLIMP-1, but high levels of KLF2, EOMES, and TCF1 (encoded by *Tcf7*).



T effector memory ( $T_{EM}$ ) cells compared to  $T_{CM}$  cells express higher levels of the TFs T-Bet, ID3, and Blimp-1 and down-regulate EOMES and TCF1 (Billingsley et al. 2015). Therefore,  $T_{EM}$  cells down-regulate the homing receptors CD62L and CCR7 and are able to patrol the blood and non-lymphoid tissues. In contrast to  $T_{CM}$  cells, they have a reduced capacity to produce IL-2 and undergo self-proliferation, but sustain high levels of cytotoxic molecules and effector cytokines (Bouneaud et al. 2005). This is important during a recall response, once known pathogens invade the body again,  $T_{EM}$  cells confer a rapid response resulting in efficient pathogen elimination (Chen et al. 2018).

T resident memory ( $T_{RM}$ ) cells became known as non-circulating T cell populations that reside in the tissue. They were discovered less than 15 years ago as T cells and were shown to outnumber largely T cells found in SLOs or blood circulation (Gerbhardt et al. 2009).  $T_{RM}$  cells adopt a unique transcriptional program allowing them to survive in the local environment. Hobit and Blimp-1 are signature TFs of  $T_{RM}$  cells in mice, acting synergistically to downregulate Eomes and Klf2, but keeping the intermediate expression of T-bet (Mackay et al. 2016). This is important as Klf2 induces CCR7 expression, leading to retention in SLOs (Skon CN et al. 2013). Nevertheless, intermediate expression of T-bet is needed to sustain IL15R $\alpha$  expression important for  $T_{RM}$  long-term survival (Mackay et al. 2015). Furthermore, another transcription factor, Runx3, is induced by TGF $\beta$  signaling (Krishnan et al. 2017) and represents an important TF for CD8  $T_{RM}$  cells, which induce the expression of CD69, CD49a, and CD103 (Milner et al. 2017). CD69 is a C-type lectin antagonizing the expression of Sphingosine-1-phosphate receptor 1, and thus promoting residency by interfering with tissue egress (Mackay et al. 2015). CD103 is the  $\alpha E$ -Integrin that forms a heterodimer with  $\beta 7$  integrin and binds mainly to E-Cadherin being important for tissue retention (Zhang N et al. 2015). CD49a binds to collagen and can therefore anchor the T cells in the extracellular matrix of the tissue (Melssen et al. 2021). In addition, Runx3 together with Bhle40 shapes  $T_{RM}$  cells' metabolic program to use mainly fatty acid oxidation, which helps  $T_{RM}$  cells to survive under conditions with low glucose and oxygen levels (Pan Y et al. 2017). The above findings are summarized in **Fig. 1.4**.



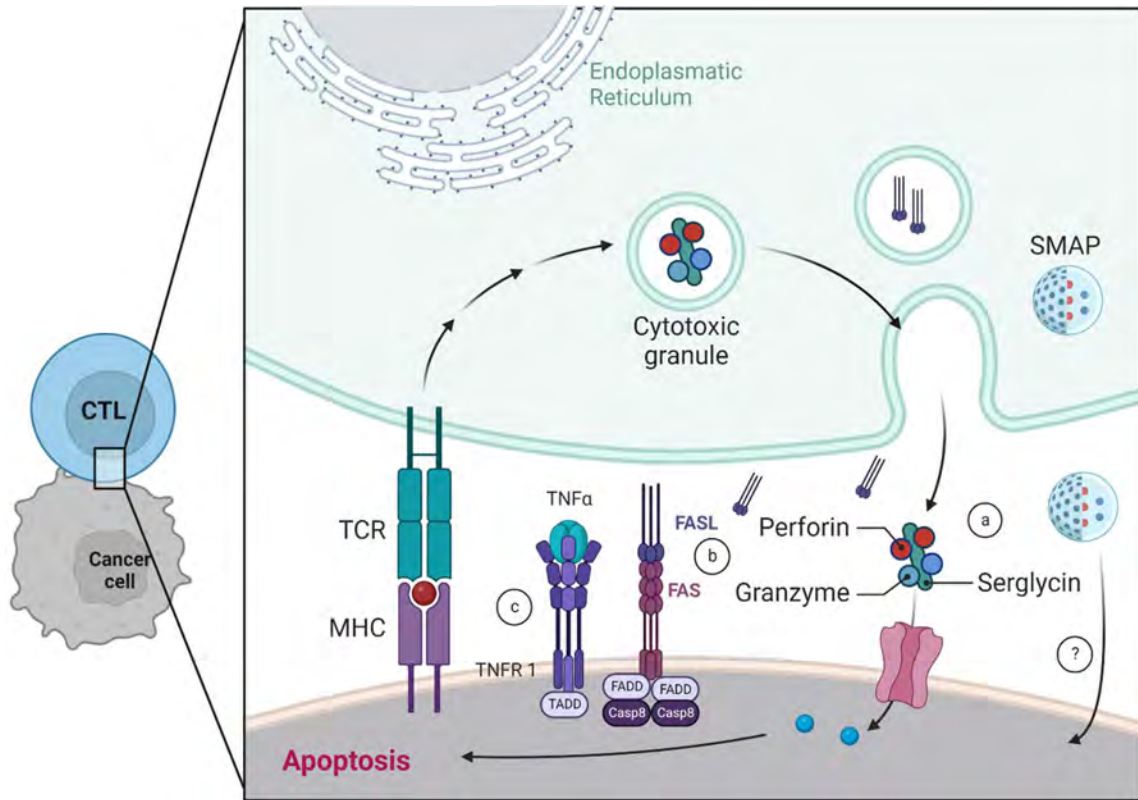
**Figure 1.4 Transcription factors and cytokines that drive CD8 T cell memory subsets of the Type I response.** After activation by cDCs, T cells receive various signals (i.e. cytokines) that drive the expression of mainly T-Bet, Blimp1, and Id2. During the effector-to-memory transition, the balance and expression patterns of TFs change, depending on the T cell memory subset. TCM express high levels of EOMES, TCF1, and KLF2 and remain in second lymphoid organs due to CCR7 expression. TEM sustains high levels of T-bet and Blimp1 and expresses Id3, which confers to rapid effector functions. They circulate in the blood and can enter into non-lymphoid organs. TRM is formed upon antigen reencounter in the tissue and expresses the integrins and surface markers CD49a, CD103, and CD69 under the transcriptional control of Hobit, Blimp1, and Runx3, which make them anchor in the tissue.

T cell differentiation into memory subsets depends on TCR signal strength, costimulatory signals, and inflammatory signals i.e. cytokines, therefore the above-mentioned TFs are mainly restricted to type I immune responses. For example, other cytokines such as IL-6 induce the TF ROR $\gamma$ T, which suppresses T-Bet and promotes IL-17 production in T cells. Furthermore, CD4 T cells depend on other TFs during their differentiation phase compared to the CD8 T cell, mainly ThPOK (Taniuchi et al. 2018). This might explain the functional differences between those subsets, which will be discussed in the following chapter. Further research is certainly needed to understand not only the signals important for the differentiation but also the maintenance of memory populations, which might involve TFs yet to be discovered. A large part of this Ph.D. Thesis involved the study of brain CD8 T<sub>RM</sub> cells, which will be the focus of *chapter 1.1.3*.

### 1.1.2 Role of activated CD4 and CD8 T cells within the immune response

Classically, the immune system is divided into innate and adaptive components. While innate immunity is fighting invading pathogens in an initial, rapid response, the adaptive response is established 3-4 days after leads to a specific and efficient clearance of invading pathogens and retains the memory of the first encounter with the antigen. One arm of the adaptive immune response involves B cells, which are able to produce cytokines and target-specific antibodies that can scavenge pathogens or particles such as viruses or bacteria. The other crucial component of the adaptive immune system is T cells, which are enabled via their T cell receptor (TCR) to recognize their MHC:peptide ligands very specifically and mount an effective immune response via direct killing mechanisms and recruitment of other parts of the immune system to the site of inflammation. CD8 T cells can interact with all body cells that present antigenic peptides via the MHC-I molecules, which can lead to direct cell lysis by their cytotoxic machinery. CD4 T cells on the other hand stand in interaction with specialized antigen-presenting cells (APCs) that present their antigenic peptides via the MHC-II molecules and create cytokine and chemokine gradients directing other immune cells to the side of infection (Samji et al. 2017).

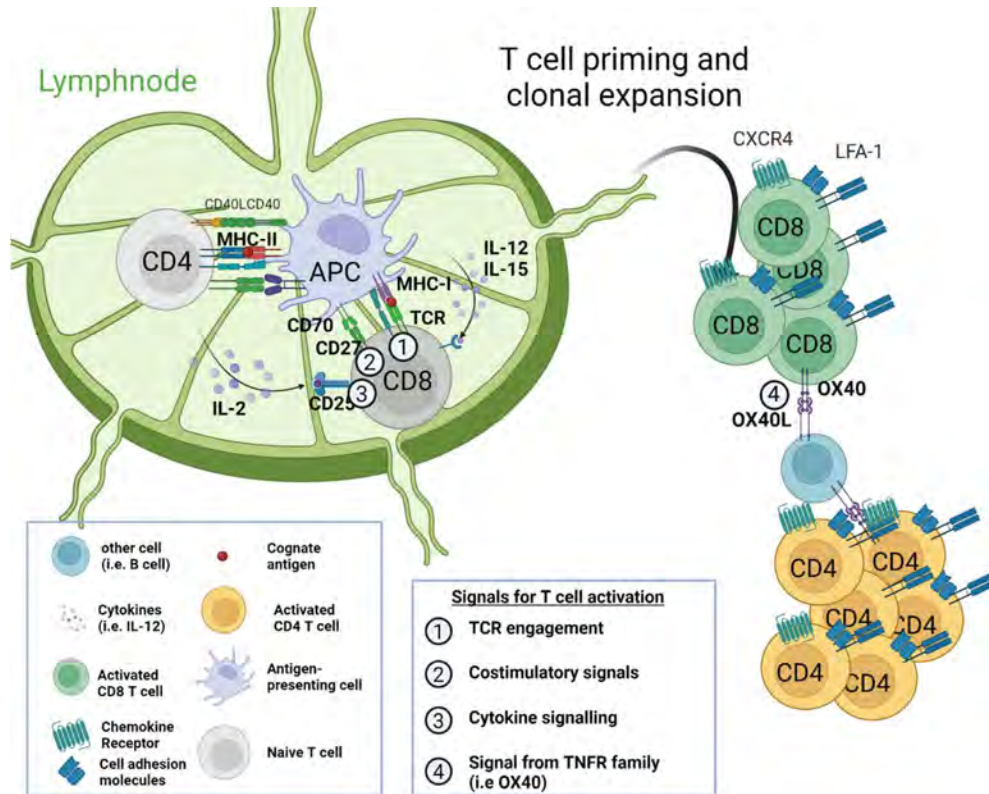
CD8 T cells are important for controlling threats to host survival that come from within, namely intracellular pathogens and cancerous growths. When a naïve CD8 T cell encounters a foreign antigen in the context of MHC-I, it undergoes clonal expansion and forms a pool of effector T cells ( $T_{EF}$ ; details in 1.2.2). Upon antigen recognition, these  $T_{EF}$  are able to produce cytokines, such as  $IFN-\gamma$  and TNF, which repress viral replication and directly lead to the death of cells expressing the TNF receptor (Park et al. 2014). Besides, CD8 T cells can release cytolytic molecules such as Perforin and Granzyme B from intracellular stores by which they can directly lyse their target cells (Wherry et al., 2003). These intracellular stores are termed lytic granules, which keep their granzymes in an inactive state by the low pH, which also inhibits the Perforin monomers from polymerizing and forming membrane pores. Once a CD8 T cell encounters its target cells upon antigen recognition via MHC-I, it forms an immunological synapse (IS) where they are polarized towards the synapse, released in the cleft and its components are activated, leading to pore formation in the target cell and subsequent cell lysis (**Fig. 1.5**). Besides, Cytotoxic T lymphocytes (CTLs) release Fas-Ligand into the synaptic cleft of the IS, which can induce the apoptotic cascade in cells that express Fas (Cassioli et al. 2022). Recently, new supramolecular attack particles consisting of granzymes and Perforin have been discovered, which might enrich the repertoire of CTL to fight against cancer cells that try to evade cell killing through a variety of resistance mechanisms (Balint et al. 2020).



**Figure 1.5 Killing mechanisms of cytotoxic T Lymphocytes.** In contact with a cell that expresses the cognate antigen, a CTL forms an immune synapse with the target cell. Following this, lytic granules are released in the cleft which contains perforin monomers stabilized by Serglycin and Granzymes. Due to the change in the pH, the Perforin monomers dimerize to a pore, which enables the influx of Granzymes in the target cell (a). Further cytotoxic granules contain Fas-Ligand, which binds to Fas and induces a Caspase 8-dependent cell death of the target cells (b). Furthermore, secreted TNF can induce TNFR1-induced cell death in the target cell (c). The supramolecular attack particles exist outside of the LGs and induce a yet-to-be-understood death in their target cells.

CD8 T cell activation requires the help of CD4 T helper cells ( $T_H$ ) via “licensing” of APCs such as dendritic cells (DCs). CD4 T cell help has been shown to involve cytokine production that is recognized by DCs and CD8 T cells as well as i.e. CD40–CD40L interactions between APCs and CD4 T cells that activate the former to provide activation signals to CD8 T cells (Schoenberger et al., 1998; Wang et al., 2003). Besides the secretion of IL-12 and IL-15 by these activated APCs, CD80/CD86 and CD70 are upregulated, which provide co-stimulatory signals to CD8 T cells via CD28 and CD27 respectively (Prilliman et al. 2002; Taraban et al. 2004). These signals were shown to be important for efficient clonal expansion and memory formation of CD8 T cells. More recent studies have revealed that several effector functions of ‘unhelped’ CD8 T cells are impacted: the production of  $IFN\gamma$  and TNF (Provine et al. 2016) and the production of Granzyme B as well as their migratory properties (Ahrends et al. 2017). The reduced ability of CD8 T cells to produce  $IFN\gamma$  and TNF supports T cell exhaustion, and indeed Provine et al. were able to show that on the transcriptome and protein level, unhelped

CD8 T cells display an exhausted phenotype and were unable to clear an adenoviral infection. Ahrends et al. 2017 elucidated further that unhelped CD8 T cells were unable to invade the tumor because they lacked expression of the migratory receptors CXCR4 and CX3CR1 and metalloproteases, which seemed to be important for tissue invasion. Taken together, CD8 T cell activation requires i) antigen recognition via their TCR, ii) signals from co-stimulation (i.e. CD28), iii) cytokine signaling (i.e. IL-12) and iv) TNF receptor interactions (i.e. OX40-OX40L). The four steps of T cell activation are shown below in **Fig. 1.6**.



**Figure 1.6 Activation of T cells in the lymph nodes.** In the LNs, CD8 T cells are primed and licensed in contact with an APC. These receive activation signals from CD4 T cells to produce cytokines. The condition for correct T cell activation is the recognition of the cognate antigen by the TCR and costimulatory signals mainly by CD28/CD80-CD86 (CD4 T cells) or CD27/CD70 (CD8 T cells) interactions. Further signals are provided by the cytokines secreted by activated DCs. Besides, further signals that sustain the survival and differentiation of activated T cells are provided from TNF- receptor superfamily members(TNFSR) such as the Ox40-Ox40 Ligand interaction depicted here. These are often referred to as environmental cues.

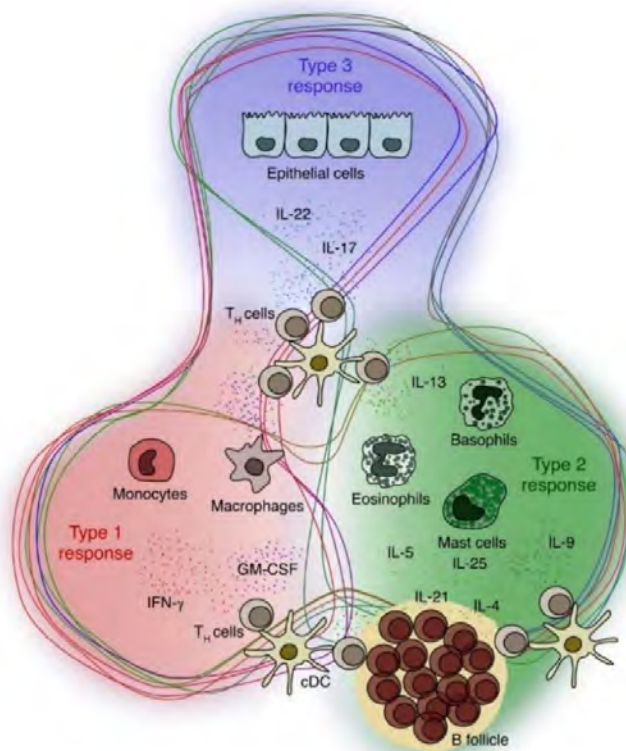
Upon antigen recognition, CD4 T cells can differentiate into a variety of effector subsets, including  $T_H1$ ,  $T_H2$ , and  $T_H17$  cells, follicular helper T ( $T_{FH}$ ) cells, and induced regulatory T ( $iT_{REG}$ ) cells (Zhou, Chong, & Littman, 2009) that were defined based on the cytokine they produced and unique transcription factors (Zou et al. 2010). Naïve CD4 T cells differentiate into those subsets depending on several parameters including antigen density, TCR affinity for the recognized MHC:peptide complex, and, probably most importantly, the cytokines present in the microenvironment at the time of priming. Since the use of high-dimensional flow cytometry and single-cell RNA sequencing (scRNAseq), the stratification of  $T_H$  cells in subsets becomes more and more doubtful, due to the high diversity ( $T_H$  cells producing simultaneously several cytokines) and plasticity (i.e.,  $T_H17$  cells can convert into  $T_H1$  cells during disease course). Therefore, the field tends more and more to refer to  $T_H$  cells of type I, II, and III responses, which those CD4 T cells support (Tuzlak et al. 2021).

Type I responses are characterized by the cytokine IFN- $\gamma$  and GM-CSF, which are involved in cellular immunity against intracellular pathogens (Mosmann & Coffman, 1989). IFN- $\gamma$  stimulates via the IFN $\gamma$ R1 (expressed on many cell types) and IFN $\gamma$ R2 (expressed mainly on phagocytes) MHC-I and MHC-II expression, respectively, triggering enhanced antigen-presentation. Furthermore, IFN- $\gamma$  stimulates the expression of cell adhesion molecules (VCAM-1 and CD44) to enable leukocytes to remain in the infected tissue (Hertzog et al. 2011). The GM-CSF receptor is almost exclusively expressed on myeloid cells and drives their differentiation, survival, and activation (Becher et al. 2016).

Type II responses are needed to fight pathogens that cannot be phagocytosed such as helminths and other extracellular parasites. Herein, the humoral immune system is activated (Pulendran & Artis, 2012). Classical Type II response cytokines involve Interleukin-4 (IL-4), which is needed for IgG class switching in B cells and plays therefore an important role in B cell maturation. Besides this, the physical interaction of  $T_{FH}$  cells with B cells through CD40-CD40L interactions and IL-21 production is essential for germinal center response including efficient memory B cell formation and antibody production (Eisenbarth et al. 2021). Furthermore,  $T_{FH}$  cells were shown to position B cells in tertiary lymphoid structures (TLS) via CXCL13 gradient and physical PD-1:PD-1 Ligand interactions to mount efficient local immune responses against i.e. tumors (Chaurio et al. 2022). TLS mount effective immune responses, as they allow T cell activation via APCs to take place at the site of infection and not in SLOs as described above.

Beside this, IL-5 and IL-13 are other Type II response cytokines that were shown to recruit eosinophils and basophils to the side of infection (Koyasu et al. 2011). Finally, IL-9 was shown to play a role in mast cell expansion and recruitment, involved in the defense of extracellular parasites (Townsend et al. 2000).

Type III responses are directed against extracellular bacteria and fungi, especially at mucosal surfaces and barrier tissues such as the skin (Stockinger, Veldhoen, & Martin, 2007; Zielinski et al., 2012). Key cytokines are IL-17A, IL-17F, and IL-22, which communicate with non-hematopoietic cells such as epithelial cells, endothelial cells, and stromal cells. These in return respond through the production of chemokines and cytokines to recruit other immune cells to the tissue such as was shown for massive neutrophil recruitment by keratinocytes in psoriasis (Zwicky et al. 2020). Besides the role of IL-17 in creating an auto-inflammatory syndrome, IL-17 production by CD4 T cells was shown to be crucial in the defense against pathogens that invade tissue barriers such as *Candida albicans* (Conti et al. 2014). This model is summarized below.



**Figure 1.7 Orbital model of immune responses.** After activation by cDCs, T<sub>H</sub> cells are involved in three types of responses: Type I responses defined by IFN $\gamma$  and GM-CSF lead to the attraction of monocytes, macrophages, and DCs resulting in phagocytosis. Type II responses defined by IL-4, IL-5, IL-9, IL-13, and IL-21 lead to Mast cell, Basophil and Eosinophil recruitment and effective B cell responses, mounting a humoral immunity against extracellular parasites. Type III responses defined by IL-17 and IL-22 communicate with non-hematopoietic cells, which recruit other immune cells to the site of infection. The lines indicate that T<sub>H</sub> cells, due to i) their plasticity and ii) the broad role of cytokines in activating many immune cells, can partake in not only one type of response during inflammation; *Tuzlak et al. 2021*

Nevertheless, it needs to be taken into consideration that the categorization into these three types of responses is limited given other regulatory and tissue-repair functions (such as IL-10, and amphiregulin), in which T cells contribute once the infection is cleared. Therefore, an understanding of the effect of cytokines is of greater use, as T cells switch their cytokine profile depending on their tissue microenvironment.

The interplay of CD4 T helper and cytolytic CD8 T cell responses enables the body to fight against a variety of foreign pathogens as well as cancer cells. Cancer cells arise normally in the tissue and pathogens try to enter the body via barrier tissues. Therefore, the local presence of resident CD4 CD8 T cells and innate immune cells is needed to create the first line of defense. This will be discussed in the following chapter.

### 1.1.3 Generation, maintenance, and function of $T_{RM}$ cells

$T_{RM}$  cells persist in barrier tissues, including the skin, lung, gut, and female reproductive tract, and non-barrier tissues, including the brain, kidney, pancreas, and joints.  $T_{RM}$  cells patrol tissues and scan them for cognate antigens (Beura et al. 2018). This makes  $T_{RM}$  cells an important first line of control against many pathogens, which enter the body through barrier tissues where the access of circulating memory T cells is limited. It has now become evident that  $T_{RM}$  cells largely outnumber  $T_{CM}$  and  $T_{EM}$  cells in the human body (Steinert et al. 2015).

A series of studies since the discovery of  $T_{RM}$  cells have revealed more about their generation.  $T_{RM}$  cells are terminally differentiated effector T cells, however, they are long-lived (Beura et al. 2018), do have the capacity to proliferate (Park et al. 2018), and even undergo self-renewal (Schenkel et al. 2016). An interesting study demonstrated that precursor  $T_{RM}$  cells arise from naïve CD8 T cells being activated by DCs in draining LNs, which present to them both the cognate antigen and the biologically active form of TGF $\beta$  (Mani V et al. 2019). TGF- $\beta$  seems to be a key cytokine for the maintenance of  $T_{RM}$  cells in the tissue. Another study revealed that  $T_{RM}$  cells in the brain have up to 20-fold higher TCR affinities compared to the recirculating memory pool (Frost et al. 2015). In line with this observation, the depletion of a special, cross-presenting type of DC (DNGR1<sup>+</sup> DC) impacts more strongly the generation of  $T_{RM}$  cells than of circulating memory subsets. The blockade of IL-12, IL-15, and CD24 impaired  $T_{RM}$  cell generation, suggesting that these cytokines are important during the priming phase (Iborra et al. 2016).



The question arose as to whether  $T_{RM}$  precursors exist. To address this question, Parga-Vidal et al. highlighted in 2021 the presence of  $Hobit^+$   $T_{RM}$  precursor cells in the spleen and LNs during the effector phase, which upregulated CD49a, CXCR6, P2RX7, and LFA1, but downregulated CXCR1, KLRG1, and S1PR1. Furthermore, the adoptive transfer of  $Hobit^+$  T cells revealed that  $Hobit^+$  effector cells were imprinted to differentiate into  $T_{RM}$  cells in the host liver, whereas the  $Hobit^{neg}$  T cells maintained high Eomes expression and formed very few  $T_{RM}$  cells. Taken together, this and the high affinity of the TCR towards the cognate antigen suggest that TCR signal strength, certain co-stimulatory molecules, and certain cytokines such as TGF $\beta$  cytokines might imprint the program in naïve T cells to differentiate into  $T_{RM}$  precursors.

The migration of  $T_{RM}$  cells in the respective tissue might be dependent on CXCL16 or CXCL9/10 gradients, as  $T_{RM}$  cells in various tissues express high levels of the chemokine receptors CXCR3 and CXCR6 (Mackay et al. 2013; Fernandez-Ruiz et al. 2016; Wein et al. 2019).

Furthermore,  $T_{RM}$  cells have higher expression of costimulatory molecules such as 4-1BB, ICOS, and GITR (Mackay et al. 2016). A recent publication investigated the role of ICOS-ICOSL interaction for efficient  $T_{RM}$  differentiation. ICOS deficiency strongly impaired CD8  $T_{RM}$  cell formation. They demonstrated, in elegant experiments, that ICOSL deficiency in stromal cells (but not in APCs) induced the upregulation of Klf2 and therefore the tissue exit, suggesting that ICOS-ICOSL interaction with the local tissue environment is crucial for tissue retention. Of note, blocking ICOS signaling after the priming phase did not impact  $T_{RM}$  persistence (Peng et al. 2022). The authors discussed that these interactions are important for the recognition of the cognate antigen in the tissue. In the central nervous system (CNS) local antigen re-encounter seems to be essential for effective  $T_{RM}$  cells formation (Schøller et al. 2019): Upon Adenoviral infection (Ad I), the formation of adenoviral-specific CD8  $T_{RM}$  cells was observed in the CNS only when the very same Ad (but not another Ad strain) was injected intra-cranially (i.c.) and recognized by the CD8 T cells in the tissue. The study brought evidence that although CD8 T cells are recruited to the CNS by IFN $\gamma$  induced inflammation, the formation of antigen-specific  $T_{RM}$  cells requires the recognition of the cognate antigen in the CNS.

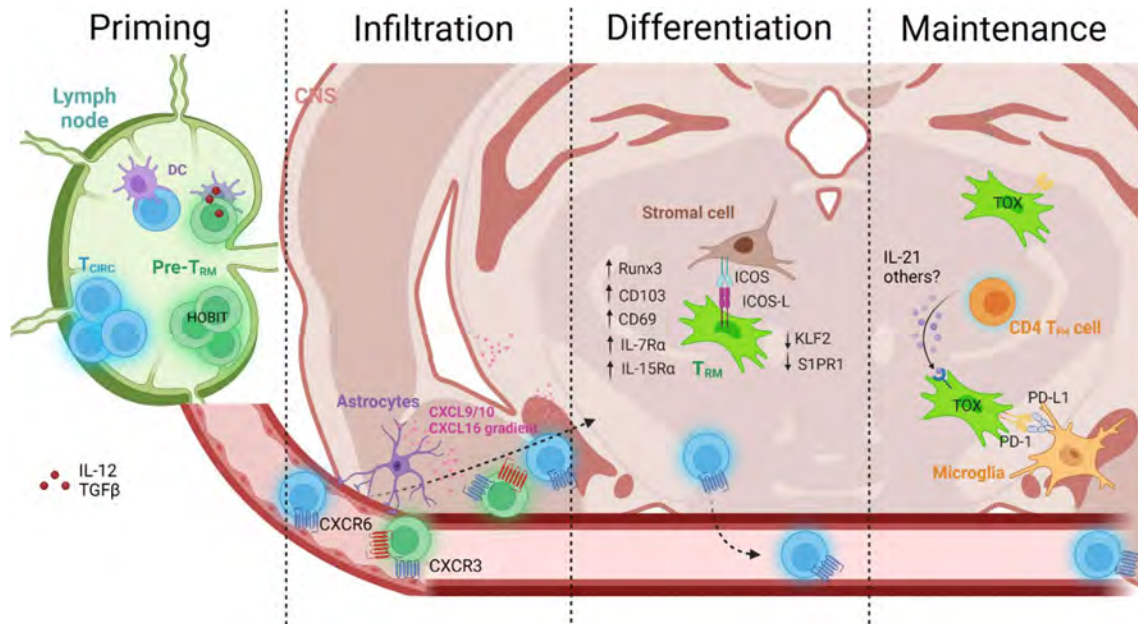
A follow-up study revealed that the local antigen re-encounter induces the expression of several  $T_{RM}$  markers important for tissue retention, such as CD103, CD69, CD44, and interestingly PD-1. PD-1 is classically considered to be an exhaustion marker associated with decreased proliferation and effector functions, but brain CD8  $T_{RM}$  cells express PD-1, LAG-3, TIM-3, and

CD39 as classical exhaustion markers. Nevertheless, these are well able to proliferate, produce IFN $\gamma$  and have high levels of Granzyme B stores. The authors suggested that PD-1-PD-1 ligand interactions might allow brain CD8 T<sub>RM</sub> cells to interact locally with microglia, macrophages, monocytes, and astrocytes (Schøller AS et al. 2021). Interestingly, brain-resident T<sub>RM</sub> cells maintain high PD-1 expression independent of the presence of their antigen, which suggests that PD-1 in the brain is not a marker for chronic antigen exposure. (Shwetank, Abdelsamed HA 2017). A human study of CD8 T<sub>RM</sub> cells in the CSF of multiple sclerosis patients revealed that PD-1 expression directly correlates with IFN $\gamma$  expression, indicating that PD-1 expression in brain CD8 T<sub>RM</sub> cells might not be an indicator of T cell exhaustion in an autoimmune context (Pappalardo et al. 2020). In line with this, brain CD8 T<sub>RM</sub> cells reactive to self-antigen require the expression of the TF TOX, which is normally associated with T cell exhaustion. Of note, genetic depletion of TOX did not impair IFN $\gamma$  or TNF production but did IL-2 production, and resulted in decreased PD-1, Lag3, and Tim3 expression and impaired CD8 T<sub>RM</sub> maintenance in the mouse CNS (Page et al. 2021). Another study by Page et al. in 2018 highlighted that TOX expression in CD8 T<sub>RM</sub> cells contributed to their encephalitogenic potential. TOX-deficient CD8 T cells displayed reduced arrest coefficients in the CNS, suggesting that they fail to recognize their cognate antigen recognition. Further, they had reduced CD107a expression, resulting in impaired astrocyte killing in a CNS autoimmune setting. CD8 T cells from MS patients but not stroke patients expressed TOX to 15-20% in the brain parenchyma (Page et al. 2018). Further studies are needed to understand why the expression of co-inhibitory molecules promotes long-term survival of autoimmune CD8 T<sub>RM</sub> cells in the brain.

Recent studies have brought evidence that T<sub>FH</sub> cells are important for the long-term persistence of CD8 T<sub>RM</sub> cells in non-lymphoid tissues. The expression of CD69 and CD103 was impaired in lung CD8 T<sub>RM</sub> cells associated with reduced persistence in the tissue (Laidlaw et al. 2014). Another study elucidated that tissue-resident T<sub>FH</sub> cells secrete IL-21, which signals via the IL-21R in CD8 T<sub>RM</sub> cells to promote their survival in the tissue (Ren et al. 2020, Son et al. 2021). This might be due to a failure in the induction of oxidative metabolism in CD8 T cells via IL-21 signaling (Ren et al. 2020). Another study, together with our own, brought evidence that in an autoimmune context of the CNS, tissue-resident T<sub>FH</sub> cells promote the persistence and cytotoxic capacity of brain CD8 T<sub>RM</sub> cells (Vincenti et al. 2022, Frieser et al. 2022).

Elevated expression of receptors for IL-7 and/or IL-15 indirectly suggests that T<sub>RM</sub> cells' long-term maintenance depends on these homeostatic cytokines (Park et al. 2018). Skin CD8 T<sub>RM</sub> cells need both IL-7 and IL-15 for long-term survival (Adachi et al. 2015), whereas intestinal

CD8<sup>+</sup> T<sub>RM</sub> cells are maintained independent of IL-15, but require TGFβ to stay for long-term in the epithelium (Zhang et al. 2013). It seems that different cytokines promote the long-term survival of T<sub>RM</sub> cells in different non-lymphoid tissues. However, which cytokine signals are important for T<sub>RM</sub> cell maintenance in other tissues, such as the CNS, is under current investigation. The above-mentioned points about T<sub>RM</sub> generation are summarized in **Fig. 1.8**.



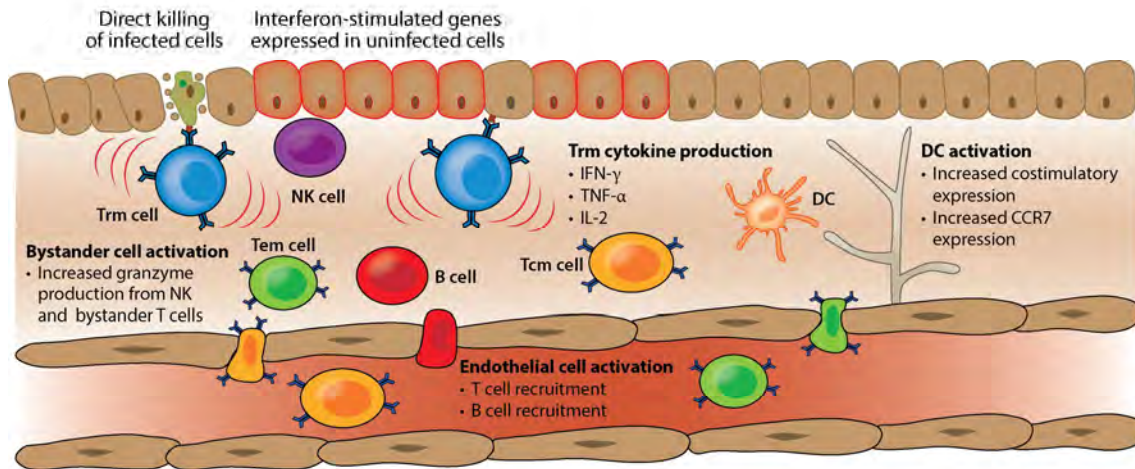
**Figure 1.8 Hypothetical Model of the generation of brain T<sub>RM</sub> cells.** Upon activation in the LNs, DCs present to naïve T cells during T cell activation the active form of TGFβ (in red), which leads to the formation of T<sub>RM</sub>-Precursors. These enter the CNS through the migration of chemokine receptors and integrins. Once in the CNS, they are stalled by local cells that express ICOS-L. Upon antigen re-encounter, the T<sub>RM</sub> differentiation program is initiated. The maintenance of T<sub>RM</sub> cells by cytokines is under current investigation, but TOX was shown to drive the expression of PD-1 being important for the long-term persistence of autoreactive CD8 T<sub>RM</sub> cells in the brain.

CD4 T<sub>RM</sub> cells share many similarities with their CD8 T<sub>RM</sub> counterparts regarding high CD69 expression, although their migration pattern and location are quite different. In the skin, CD8 T<sub>RM</sub> cells resided in the epidermis, while CD4 T<sub>RM</sub> cells were located in the dermis (Gebhardt et al. 2011). A recent study highlighted that this is due to a lower expression of Runx3 in CD4 T<sub>RM</sub> cells, which express rather the TFs Runx1 and ThPOK. The forced expression of Runx3 in CD4 T<sub>RM</sub> cells resulted in an upregulation of CD103 and CD49a due to chromatin remodeling of the loci responsible for effective TGFβ signaling. This changed the localization of CD4 T<sub>RM</sub> cells from the dermis to the epidermis (Fonseca et al. 2022). Interestingly, CD4<sup>+</sup> T<sub>RM</sub> cells in various tissues form aggregates with other resident immune subsets such as macrophages, DCs, and CD8 T cells (Gray JI et al. 2018, Collins N et al. 2016) and might therefore carry out in situ T helper functions supporting CD8 T<sub>RM</sub> cells.

T<sub>RM</sub> cell function in protection against tumors and pathogens

T<sub>RM</sub> cells have a protective role in tumor control. It was indeed shown that the number of CD103<sup>+</sup> tumor-infiltrating lymphocytes correlates with a favorable prognosis in many forms of cancer (Wang et al. 2015, Djenidi et al. 2015, Ganesan et al. 2017, Koh et al. 2017). As CD103 is binding to E-cadherin, it was argued that CD103 expression by CD8 T cells facilitates the infiltration in and even the killing of epithelial tumors (Gauthier et al. 2017). It was observed that the binding of CD103 from CD8<sup>+</sup> T cells to epithelial cancer cells expressing E-cadherin shaped the immunological synapse morphology, which was essential for polarizing lytic granules containing Granzyme B to the target cell (Franciszkiewicz et al. 2013). Although E-cadherin expression is often lost during malignant transformation and tumorigenesis, CD103 has other binding partners such as N-cadherin (Jenkinson SE et al. 2011). The key role of T<sub>RM</sub> cells in mediating anti-tumor activity was underlined by a study of B16 melanoma in CD103<sup>-/-</sup> mice, where the absence of T<sub>RM</sub> cells in the skin completely abrogated control of skin tumor (Malik BT et al. 2017).

Several studies have documented the protective role of T<sub>RM</sub> cells against all major classes of microbial pathogens: viruses, bacteria, fungi, and parasites (Gebhardt et al. 2009, Wu et al. 2014, Jiang et al. 2015, Fernandez-Ruiz et al. 2016, Connti et al. 2014, Kinnear et al. 2018). T<sub>RM</sub> cells have been shown to provide efficient long-lived immunity against pathogens that use peripheral tissues as entry sites into the body, such as HIV through the female reproduction tract (Jiang et al. 2012, Buggert et al. 2018). T<sub>RM</sub> cells possess several anti-pathogenic functions. By producing pro-inflammatory cytokines, they are able to fight incoming pathogens and alarm the neighborhood (**Fig. 1.9**). Upon recognition of their cognate antigen, T<sub>RM</sub> cells secrete IFN- $\gamma$ , IL-17, and TNF- $\alpha$ , which will firstly directly resist viral replication and secondly activate epithelial cells and dendritic cells so that more leukocytes are recruited to the infection site (Schenkel et al. 2013, Ariotti et al. 2014). Besides this, T<sub>RM</sub> cells have a high cytolytic potential due to their constitutive expression of Granzyme B (Cheuck et al. 2017).



**Figure 1.9 Effector functions of TRM cells upon antigen recognition.** When established TRM cells recognize cognate antigens, they alert neighboring cells mediated by cytokine production and kill antigen-bearing host cells. Via interferon-stimulated genes, TRM cells activate the endothelium to recruit leukocytes (local DCs, NK cells, and T cells) and induce egress of DCs to draining lymph nodes. Abbreviations: DC, dendritic cell; NK, natural killer; Tcm, central memory T; Tem, effector memory T; TRM, resident memory T. (adapted from Masopust and Soerens 2019)

#### TRM cells in autoimmune diseases

Growing evidence for the involvement of TRM cells in several autoimmune diseases is summarized in **Fig. 1.10**. CD8 TRM cells producing IFN-γ were first described in the skin autoimmune disease fixed drug eruption (Shiohara et al. 2009). After this discovery, TRM cells were also discovered in skin lesions of Vitiligo patients. These TRM cells express CD103 and CD49a and produce Perforin, Granzyme B, and IFN-γ upon *in vitro* restimulation (Cheuk et al. 2017). Furthermore, IL-17-producing CD8<sup>+</sup> TRM cells and IL-22-producing CD4<sup>+</sup> TRM cells have been described in psoriasis (Cheuk et al. 2014, Kuriharar et al. 2019). Interestingly, these TRM cells expressed the homing receptor CXCR3 sensing CXCL9 and CXCL10 (highly expressed by hair follicles), which suggests that TRM cells localization relies on chemokine gradients in the tissue (Boniface et al. 2018).

Type 1 Diabetes (T1D) is a T cell-mediated autoimmune disease characterized by the destruction of β-islet cells in the pancreas producing insulin. An enhanced frequency of CD8<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> TRM cells has been observed in insulitic lesions that up-regulate inflammatory genes including *IFNG*, *IL18*, *IL22*, and *IL15*, and chemokines including *CXCL1*, *CXCL9*, *CCL4*, *CCL5*, *CCL7*, *CCL11*, and *CCL19* (Kuric et al. 2017).

In lupus nephritis, the infiltration of lymphocytes in glomeruli partly contributes to renal damage. A recent study found an increase in CD8<sup>+</sup> TRM cells in the kidneys of patients with lupus nephritis, which correlated with the disease activity (Zhuo et al. 2020).

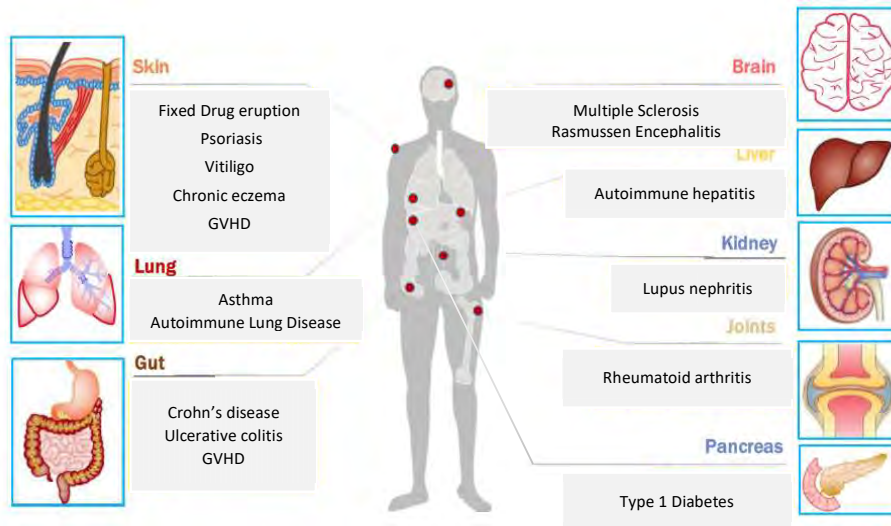


Figure 1.10 Organ-specific autoimmune diseases in which  $T_{RM}$  cells could be involved. Adapted from Wu et al. 2018.

With this in mind, the both beneficial and pathologic roles of activated memory T cells in different human CNS diseases will be discussed in the following chapters.

## 1.2 The role of activated T cells in CNS diseases

### 1.2.1 CNS diseases caused by viruses

Among others, Flaviviruses are known as neurotropic viruses that replicate in the CNS. These will be the focus of this short section on CNS diseases caused by viruses. I will also discuss recent data related to EBV and the development of MS that provide a causal relationship between viral infections pre-disposing an autoimmune disease of the CNS (molecular mimicry).

Various viral epidemics have been reported in the past 70 years and up to the present day, 400 million individuals are infected yearly reporting mild (fever, headache) to severe clinical manifestations (shock-syndrome, encephalitis, paralysis, and hepatitis) after infection with partial long-term consequences (Bhatt et al. 2013). Flaviviruses are positive-single stranded RNA-viruses that are mainly transmitted by mosquito bites and enter humans in a variety of cell types via interaction with TIM-1, Mannose receptor, and DC-SIGN depending on the strain and type of virus. They can be divided into viruses that cause visceral disease (i.e. hepatitis) to which Dengue, ZIKA, and Yellow fever viruses belong, and neurotropic viruses (i.e. encephalitis) to which ZIKA, John Cunningham virus (JCV), Japanese encephalitis (JE) and West Nile fever virus (WNV) belong. The global distribution is shown in **Figure 1.11**:

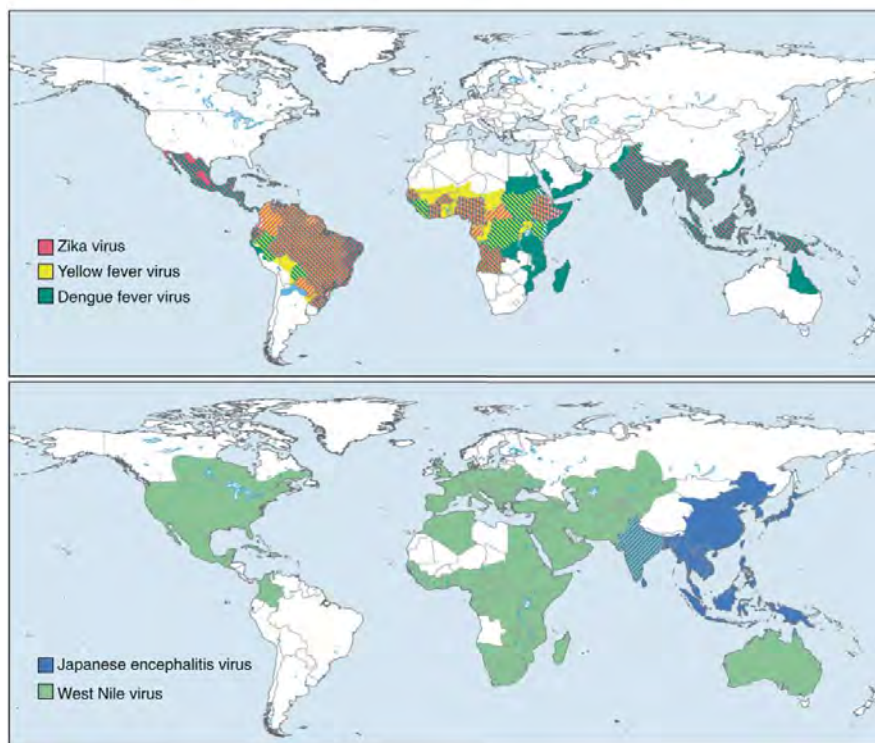


Figure 1.11 Distribution of Flaviviruses in the world. Pierson et al. 2020

The threat of John Cunningham virus, or JC virus (JCV), causing Progressive Multifocal Leukoencephalopathy (PML), has become notable since the worldwide spread of the human immunodeficiency virus (HIV) pandemic. Due to the immunodeficiency caused by the depletion of CD4 T cells in the host, JC viruses could replicate in the CNS and could no longer be controlled by the host. Furthermore, immunosuppressive treatments during T cell leukemia and Natalizumab treatment for MS patients lead to an emerging spread of JCV in the CNS, which exists in latent forms and are normally controlled in immunocompetent individuals (Cortesse et al. 2021). JCV damages the material (myelin) that covers and protects nerves in the white matter of the brain where several lesions occur, characterized by neuronal loss, which ranges from millimeters to centimeters in size depending on the viral spread. These are found mainly in the cerebral hemispheres (Bauer et al. 2015). T cells are present at the border and inside active PML lesions, wherein CD8 T cells are pre-dominant and are attributed an important role in viral clearance (Wütrich et al. 2006). In the blood of surviving PML patients both CD4 and CD8 T cells can be detected, producing mainly IFN $\gamma$ , which seems to be an important cytokine in the clearance mechanism of JCV (Gheuens et al. 2011).

JE viruses infect astrocytes and oligodendrocytes and also are controlled by IFN $\gamma$ . Turtle et al. showed in 2016 that in 39 JEV-recovered patients compared to 51 controls, the pre-dominance of JEV-specific T cells in the blood represents CD4 T cells, which produce mainly IFN $\gamma$  (Kumar et al. 2004).

A recent study highlighted that T cells in ZIKA and WNV infection trigger synaptic loss and cognitive dysfunction via IFN $\gamma$  signaling, as IFN $\gamma$  or IFN $\gamma$ R1 deficient mice don't develop cognitive dysfunctions (Garber et al. 2019). In line with this, IFN $\gamma$  production by CD4 T cells was shown to be important in the clearance of the Zika virus in the brain (Lucas et al. 2018). Interestingly, this study highlighted that IFN $\gamma$ R-deficient mice were unable to clear the virus – probably due to impaired signaling in phagocytes and other lymphocytes.

Importantly, mice depleted for CD4 T cells died faster than CD8 T cell-depleted mice. The authors brought evidence that CD4 T cells were involved in B cell maturation and antibody production against the Zika virus. High-affinity antibodies against virion structures such as the capsid and the envelope are also important in humans for viral clearance. It was suggested that virus-specific T<sub>FH</sub> cells support B cell maturation and plasma blast development against viral particles of ZIKA viruses (Koblichke et al. 2018).

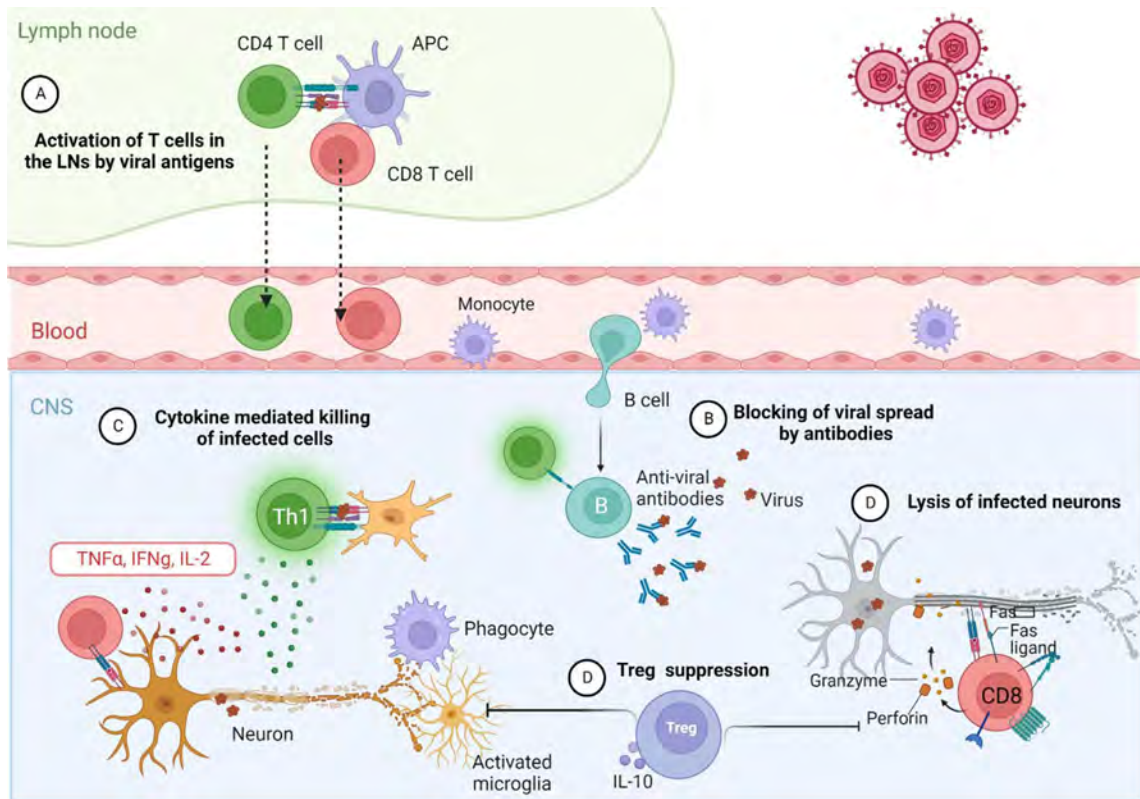


In WNV infection, CD4 T cells persist in the brain for up to 16 weeks (Stewart et al. 2011). The depletion of CD4 T cells results in the failure of the control of WNV load in the brain, but not in the spleen (Shrestha et al. 2008). A possible explanation for this is that CD4 T cells have an important role in maintaining CD8 T<sub>RM</sub> cells in non-lymphoid tissues, as discussed before. Indeed, recent studies have shown that systemic CD4 T cell depletion causes the loss of brain CD8 T<sub>RM</sub> cells (Mockus et al. 2018). The loss of brain CD8 T<sub>RM</sub> cells resulted in higher viral loads in the brain.

Besides the protective role of T cells involved in viral clearance, they also display pathogenic roles in CNS viral diseases, depending on the viral load in the CNS. One study nicely showed that CD8 T cell-depleted mice inoculated with a low titer of WNV showed decreased survival, while those with a high titer of WNV showed increased survival (Sretzer et al. 2012). Therefore, the abundant damage mediated by CD8 T cells can lead to death, which is probably mediated by the lysis of virus-infected CNS cells. In line with this, Ireland et al. showed in 2017 that the brain of mice infected with a high viral load of WNV abounds with CD8 T cells that induce massive neuronal apoptosis, leading to mortality. Mice depleted for CD8, but not CD4 T cells, showed increased survival.

Both in mice and humans, Treg cells have a beneficial role in controlling WNV infections. Symptomatic patients with headaches, weakness, or body aches have lower numbers of Treg cells in the blood compared to asymptomatic patients. In mice, Treg depletion leads to a lethal outcome in WNV-infected mice (Lanteri et al. 2009). Although one might think that the absence of Tregs would improve viral killing, their presence seems to be essential to limit the hyper activation of the immune system.

The findings regarding the beneficial and pathological effects of both B and T cells during virus infections in the CNS are summarized in the graphic below (**Fig. 1.12**).



**Figure 1.12 Involvement of T cells in the defense against neurotropic viruses.** Once DC present viral antigens that T cells recognize, the subsequent activation leads to transmigration to the CNS, wherein T cells play different roles: CD4 T cells can interact with B cells to support effective antibody production that limits the spreading of the viral particles (this already takes place in the periphery but can be reinforced in the CNS). CD4 T cells can further interact with Microglia presenting viral antigens, which leads to the secretion of type 1 cytokines. CD8 T cells that recognize infected cells such as Neurons can either directly kill them via lytic granules or recruit phagocytes that are involved in synaptic pruning. Therein, Treg cells control the fine balance of the immune response to prevent extensive tissue damage.

The Epstein Barr Virus (EBV) infection caught special attention this year because of two reports in Science and Nature investigating the causal link between EBV infection and the development of Multiple Sclerosis (MS; *see 1.2.2*). EBV was shown to be present in active MS lesions in histopathological studies (Serafini et al. 2004, Hasani et al. 2018, Moreno et al. 2018). Bjornevik et al. used a longitudinal study of US militaries from whom blood samples were systematically taken and compared militaries who developed MS later on with healthy sex- and age-matched controls. They were able to show that 97% of MS patients showed seroconversion (appearance of antibodies against EBV) compared to 57% in controls, and this happened shortly before the onset of MS (Bjornevik et al. 2022). Therefore, the risk of developing MS increases more than 30-fold after EBV infection, and one can almost say that EBV infection is a necessary (but by far not sufficient) condition to develop MS.

A potential mechanistic explanation was provided by the study of Lanz et al. who found in the CSF of MS patients clonal expanded B cell populations with specific B cell receptors. From those receptor sequences, they developed functional antibodies and found those antibodies to recognize EBV, in particular the EBNA1<sub>AA365-425</sub> epitope. Surprisingly, this antibody is recognized as well as peptides from Glial CAM protein, which is specifically expressed in astrocytes and oligodendrocytes – therefore providing evidence of molecular mimicry that was present in 25% of MS patients. Finally, immunization with EBNA1<sub>AA365-425</sub> could exacerbate the severity of EAE induced by a myelin antigen, giving a strong causal relationship between EBV infection and the development of MS.

Of note, more MHC-I–restricted EBV-specific TCR $\beta$  sequences were detected in the blood of MS patients as compared to healthy controls. Genetic predisposition was excluded since a similar observation was found in MS-discordant monozygotic twins. The data suggest that MS patients exhibit an on-going T cell response against EBV, whose role in the pathophysiology would require further analyses (Schneider-Hohendorf et al 2022). Besides, an expanded TCR clone seen specifically in the CSF of Alzheimer's Disease patients also recognizes EBV epitopes, which suggests that this virus may contribute to the triggering of other neurodegenerative diseases (Gate et al. 2020).

### 1.2.2 Multiple Sclerosis

Multiple sclerosis (MS) currently affects 2.8 Million individuals worldwide and represents a potentially disabling disease of the brain and spinal cord (Walton et al. 2020). During the disease course, 85% of the patients initially present a relapsing-remitting form of MS (RRMS) with phases of disease stability (remission) and relapses marked by weakness, sensory abnormalities, balance impairment, and visual impairment (vision loss, double vision) which last for days or weeks and can be manifested up to 1-2 times per year (Lublin et al. 2003). After each relapse, symptoms increase stepwise, and untreated RRMS will later develop in 19 years on average into a secondary progressive form of MS (SPMS, Rovares et al. 2009), where the disability increases incrementally. 10-20% of patients have a form of primary progressive MS, in which the clinical manifestations increase progressively from the start without clear-cut relapse and remission phases (Klineova et al. 2018)

MS is characterized by lesions of the brain and spinal cord and optic nerves, which are the result of demyelination of axons, neuronal synapsis loss, and astrogliosis (Wegner et al. 2006). The pathology is clinically measured by magnetic resonance. This reveals that MS patients contain several lesions (“plaques”) in the white matter and grey matter, often close to ventricles

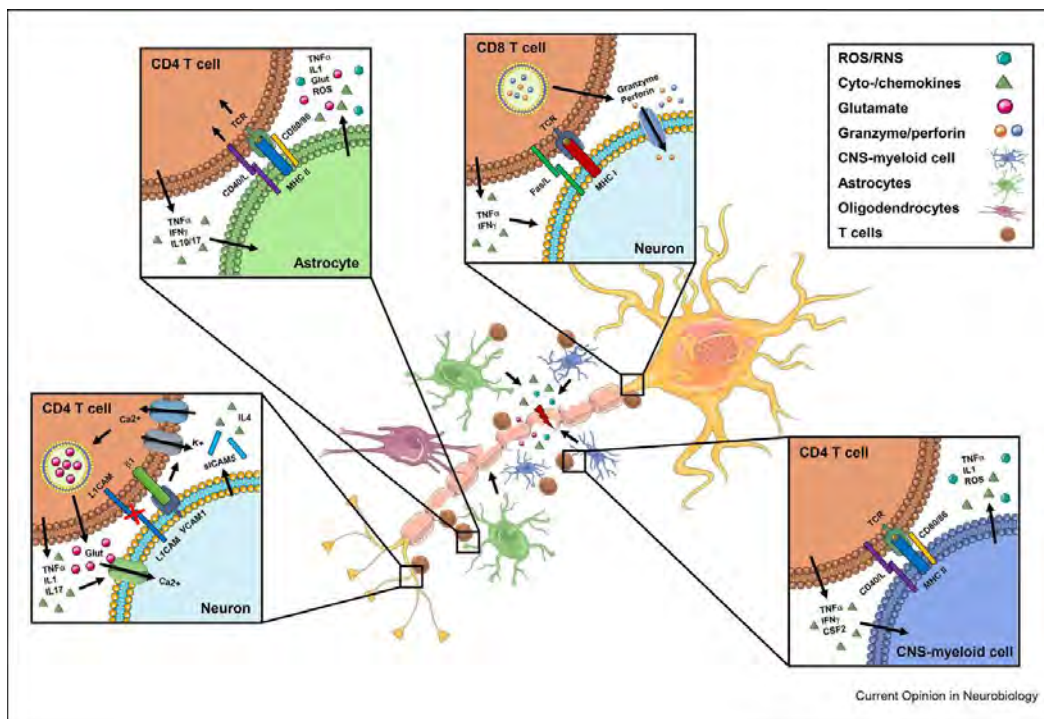
containing the choroid plexus, which is a structure of the blood-CSF barrier (Tarlington et al. 2019). This suggests that the pathology is caused by immune cells coming from the periphery. Another strong point for this hypothesis is given by the finding that subpial cortical lesions seen in MS patients with progressive disease course stand in strong spatial correlation with T and B cell accumulation in the leptomeninges, which present a common entry site for leukocytes in the CNS (Ahmed et al. 2022). Besides this, in the spinal cord, an axonal loss of motor- and inter-neurons is observed in the upper cervical region, which might explain the motor dysfunction symptoms observed during MS (Schirmer et al. 2009; Gilmore et al. 2009).

Data from animal models strengthened the idea that autoreactive T-cells are the initiators of neuroinflammation in MS pathology. The classical animal model of MS, experimental autoimmune encephalitis (EAE), is not dependant on CD8, but on CD4 T cells, probably because the myelin peptides used for immunization (such as the MOG<sub>35-55</sub> peptide) are MHC-II restricted and do not elicit myelin-specific autoreactive CD8 T cells. In the EAE model induced by MOG<sub>35-55</sub> in C57Bl/6 mice, it was shown that the immunization with the auto-antigen creates a mixed T<sub>H1</sub>/T<sub>H17</sub> response that migrates to the CNS, Th1 migrating preferentially in the spinal cord while T<sub>H17</sub> preferentially locate in the brain. In the CNS they create local inflammation resulting in clinical manifestations such as paralysis. Hallmarks of this model include a breakdown of the BBB, which leads to the infiltration of other T cells, B cells, and Myeloid cells into the CNS with the activation of resident glial cells such as microglia and astrocytes (Glatigny et al. 2018).

In the lesions of MS patients, T cells were shown to have several interactions with other immune cells, such as B cells, myeloid cells, and astrocytes. An analysis of chronic active MS lesions revealed recently that these lesions consist of T cells and DCs, suggesting a process of re-activation by antigen presentation next to MS lesions (Absinta et al. 2021). The activation of astrocytes and subsequent release of pro-inflammatory cytokines that open the BBB and neurotoxic molecules seems to be induced mainly by T<sub>H17</sub> cells, as both of these pathogenic processes are initiated via the IL-17 receptor signaling in Astrocytes (Elain et al. 2014; Wheeler et al. 2020). Another study highlighted that microglia in early MS lesions express high levels of cytokines (TNF, IL1 $\beta$ ), chemokines (CXCL16), and genes related to phagocytosis. Furthermore, the upregulation of MHC-II, CD40, and CD86 suggests that these microglia have the potential to productively interact with CD4 T cells (Zrzavy et al. 2017).

It is becoming increasingly accepted that following the adaptive immune system, the innate immune system plays a pathogenic role in the progressive phase of MS. Microglia are present in MS lesions, being close to excitatory CUX2<sup>+</sup> projection-neurons with the phagocytosis of myelin particles in upper-cortical layers. This suggests that microglia are actively involved in neuronal damage during MS pathology (Schirmer et al. 2019).

CD8 T cells can directly interact with neurons and can induce neurotoxicity i.e. mediated through polarization of Lytic granules, which can be partially observed in CD8 T cells in SPMS (Machado-Santos et al. 2018). Interestingly, T<sub>H</sub>17 cells were also shown in MS patients to directly interact with oligodendrocytes via the CD29/β1 integrin and induce the death of oligodendrocytes *in vitro* (Larochelle et al. 2021). Furthermore, the β1 integrin expressed by T<sub>H</sub>17 cells was shown to interact with VCAM-1 expressed on neurons, which would argue that CD4 T cells, despite the lack of MHC-II molecules on neurons, can indeed be in contact with neurons under some circumstances and could contribute to the demyelination and neuronal loss in the disease progression (Birkner et al. 2020). These pathogenic mechanisms of T cells in interaction with neurons are summarized in **Figure 1.13**.



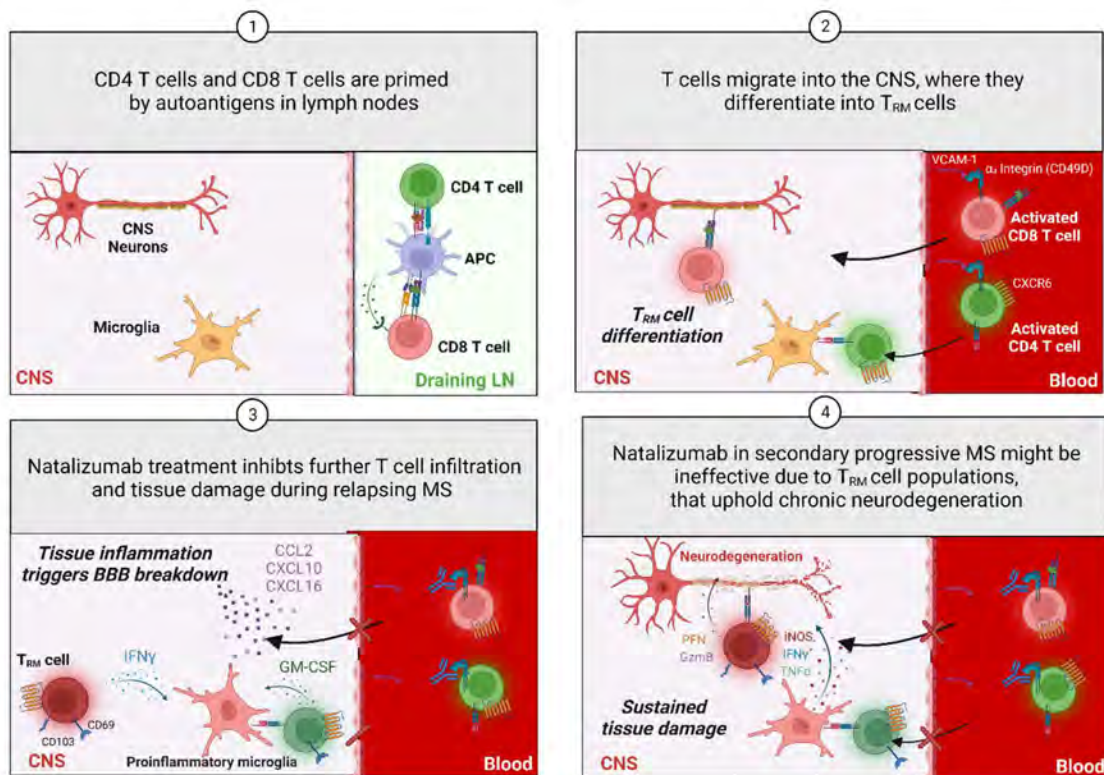
**Figure 1.13 Direct and indirect T cell–neuron interactions in MS.** After activation by CD4 T cells (via pro-inflammatory cytokines), CNS-myeloid cells and astrocytes secrete pro-inflammatory cytokines, ROS/RNS, and glutamate, causing neuroaxonal damage. Interaction of  $\beta$ 1 integrin on T cells and VCAM1 on neurons can trigger glutamate exocytosis and consequently neuronal excitotoxicity. CD8 T cells can directly interact with neurons via MHC1-TCR and Fas/FasL contacts. After MHC1-TCR interaction, CD8 T cells can damage neurons via Granzyme and perforin or pro-inflammatory cytokine secretion (Brummer et al. 2022).

A recent study aimed to find auto-antigens for MS. Autoreactive CD4 and CD8 T cells against FABP7, PROK2, RTN3, SNAP91, MOG, and PLP were identified, which are exclusively expressed in the brain. Nevertheless, the response to these antigens did not correlate with the disease progression, onset, or Natalizumab treatment (Bronge et al. 2022). This speaks in favor of this response being a consequence, rather than the cause, of CNS damage.

Machado-Santos et al. showed in 2018 that the disease duration correlated with an accumulation of CD8 T cells that expressed PD-1 and CD103, defining them as classical CD8 T<sub>RM</sub> cells that accumulate in the disease course. A recent study characterized the cellular composition of MS lesions and found significantly more T cells in the white matter of MS patients with signs of demyelination, compared to normal appearing white matter. Characterizing the CD8 T cell compartment, they found an expression of CD69, CD44, PD-1, and CD103 as classical T<sub>RM</sub> markers together with CXCR6 and GPR56, which might regulate their migration to neurons via CXCL16 gradients and transfer to neurotoxicity, respectively. Ki-67 expression in more than 25% of T cells of active lesions suggested continuous antigen recognition. Indeed, they found T cells to be in contact with myeloid cells, which may speak in favor of this hypothesis (Fransen et al. 2020).

A follow-up study found another marker, CD20, to be weakly expressed in a proportion of CD8 T<sub>RM</sub> cells that showed high expression of CXCR6, Ki-67, and Granzyme B. This population of CD20<sup>dim</sup> T<sub>RM</sub> cells is enriched in MS lesions compared to control tissues. This suggests that the anti-CD20 monoclonal antibodies used for the treatment of MS patients could target cells other than B cells that might play a role in the disease pathology (Hsiao et al. 2021). Besides in the CNS, clonally expanded CD8 T<sub>RM</sub> cells were also identified in the CSF in a study comparing six MS-discordant monozygotic twin pairs. 29% of the CD8 T cells in the twin with MS (compared to 2% in the healthy twin) were clonally expanded (9% for CD4 T cells) and the expanded clones showed high expression of pro-inflammatory cytokines such as IFN $\gamma$ , cytotoxic molecules, chemokine receptors (CXCR6 and CCR5) and HOBIT with Id2 expression suggesting that the CSF of MS patients contains clonally expanded CD8 T<sub>RM</sub> cells (Beltran et al. 2019). Pappalardo et al confirmed in 2020 this cytotoxic phenotype of CD8 T<sub>RM</sub> cells in the CSF of MS patients compared to controls (they also found them to express Perforin). Of note, CSF-derived CD4 T cells also displayed a T<sub>RM</sub>-like phenotype with the expression of *Cd69* and *Junb*.

The exact contribution of  $T_{RM}$  cells in the immunopathology of MS remains to be determined, but their presence might explain why systemic administration of monoclonal antibodies (Natalizumab) aiming at preventing immune cell transmigration from blood to the CNS appear to be ineffective in the later stage of disease progression. These CD8  $T_{RM}$  cells may sustain inflammation of the CNS independently from immune cells coming from the periphery. This hypothesis is summarized in **Figure 1.14**.



**Figure 1.14 Hypothetical model of why drugs blocking T cell entry are ineffective in secondary progressive MS. (1)** APCs in the draining lymph node present yet unknown autoantigens to T cells. **(2)** Their activation leads to the upregulation of chemokine receptors and integrins (i.e. CD49D), which enable them to transmigrate to the CNS. Therein T cells differentiate partially into  $T_{RM}$  cell roles. **(3)** Cytokine production from autoreactive T cells creates a pro-inflammatory environment, which activates resident glial and endothelial cells and creates chemokine gradients, which then subsequently leads to the breakdown of the BBB. MS patients experience in the beginning relapses, where the treatment of Natalizumab hinders new T cells from the periphery entering the CNS. **(4)** In secondary progressive MS forms, a substantial number of autoreactive T cells have acquired a tissue-resident phenotype and can uphold chronic tissue damages independently of T cells from the periphery. Neurodegeneration can be due to cell lysis of Oligodendrocytes, Neurons by cytotoxic mechanism mediated by CD8 T cells or secretion of neurotoxic factors from pro-inflammatory microglia, that might also phagocytose neuronal synapses, as some studies have shown.

### 1.2.3 Parkinson's disease

Parkinson's disease (PD) is caused by dopaminergic neuron loss, mainly in the substantia nigra, following the inclusion of body formation of  $\alpha$ -Synuclein. The clinical symptoms consist of tremor, bradykinesia and rigidity, which can be associated with cognitive defects (Jankovic et al. 2008). Early studies of the post-mortem brain of PD patients revealed activated microglia along with CD4 and CD8 T cell infiltration (but not B cells or NK cells) in the substantia nigra (Brochard et al. 2009). Therefore, it was believed that Parkinson is a neurodegenerative disease involving, as a primary or secondary event, the adaptive and innate immune system.

This notion is further supported by the identification of susceptibility genes for PD that are involved in antigen presentation to T cells. A GWAS study revealed that certain human leukocyte antigen (HLA) haplotypes in PD patients are positively associated with the disease, namely *B\*07:02*, *C\*07:02*, *DRB5\*01*, *DRB1\*15:01*, *DQA1\*01:02* and *DQB1\*06:02*. This might indicate that certain antigens presented by those HLA-molecules to CD8 and CD4 T cells are involved in the disease course (Wissemann et al. 2013). Furthermore, *Parkin* and *PINK1* gene polymorphism were found in PD patients, which suppress antigen-presentation of mitochondrial proteins and may lead to a presentation of an auto-antigen (Matheoud et al. 2016). Another gene polymorphism in the *LRRK2* gene is involved in T cell development, leading to an increase of Th17 populations in PD patients. This suggests the involvement of certain T cell populations in the disease course (Park et al. 2017).

Several studies have underlined the change of T cell compartments in the blood of PD patients. Consistent studies have found a skewing of CD4 T helper populations in the blood towards an increase of Treg cells and memory Th1 and Th17 cells compared to healthy controls (Bongioanni et al. 1997; Rosenkranz et al. 2007; Saunders et al. 2012; Chen et al. 2014; Chen et al. 2017). Activated memory CD8 T cells were also decreased in correlation with the onset of the disease (Bhatia et al. 2021). Besides this, they displayed higher expression levels of MMP9, CCR1 and CXCR3, suggesting changes in migration patterns and tissue invasiveness of CD8 T cells in PD patients.

Recent studies were able to show the response of CD4 and CD8 T cells from PD patients to  $\alpha$ -Synuclein. Sulzer et al. linked the  $\alpha$ -Synuclein peptides recognized by these autoreactive T cells to HLA-alleles and found a prevalence of *DRB1\*5:01*, *DRB5\*01:01*, *DQB1\*03:04* and *A\*11:01* for PD patients compared to healthy controls. This partially confirmed the genetic predisposition of certain HLA molecules of PD patients on the protein level (Sulzer et al. 2017).



A follow-up study showed that  $\alpha$ -Synuclein reactive T cell clones producing IFN $\gamma$  were the highest in PD patients with recent onset of motor impairments, and declined in the years after onset, suggesting that these autoreactive T cells might play a role in the initiation of the disease (Lindestam Arlehamn et al. 2020). In this study, they analyzed PBMC samples from one patient before and after disease onset and found that  $\alpha$ -Synuclein reactive T cells were present prior the disease onset, and dropped shortly after disease onset associated with shingles infection. This finding could lead to the hypothesis that the viral infection triggered  $\alpha$ -Synuclein reactive T cells to migrate into the CNS, where they later destroyed the dopaminergic neurons.

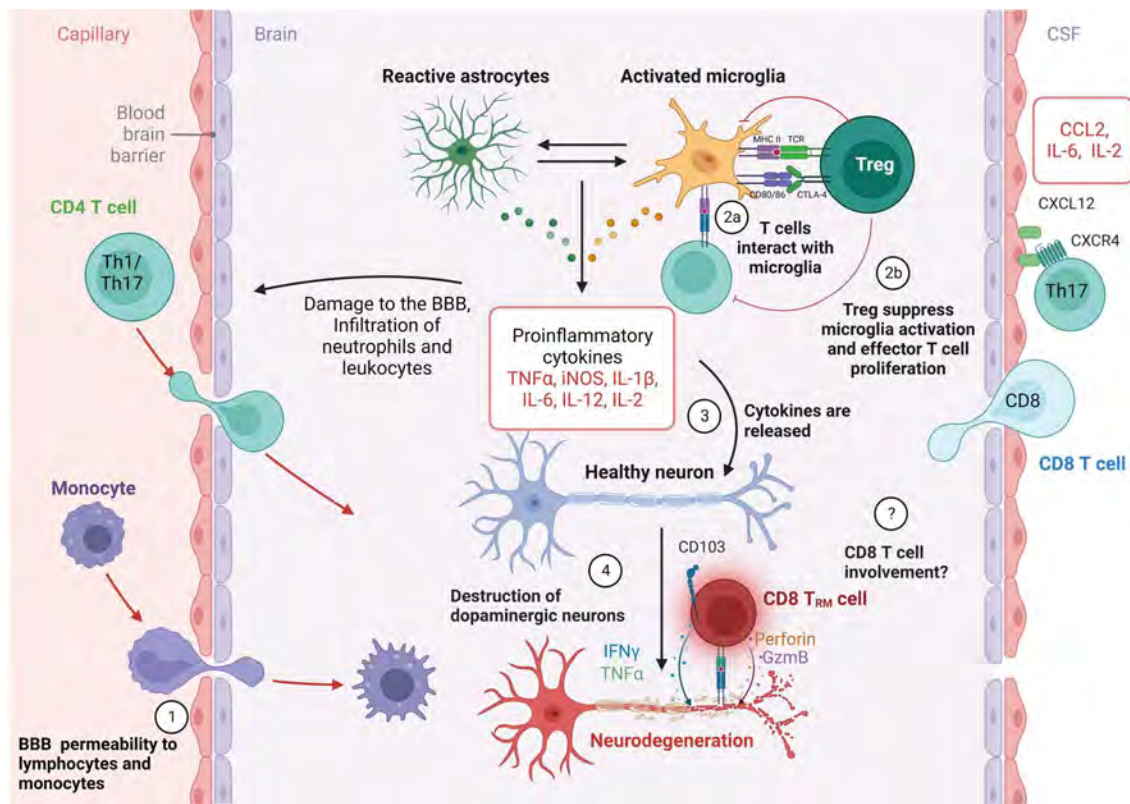
Other studies have aimed to analyze the immune composition of the CSF of PD patients. They found an increase in total T cells in the CSF, wherein CD8 T cells especially displayed an activated phenotype. Furthermore, the protein levels of IL-2, IL-6, TNF, and CCL2 in the CSF were increased, suggesting that type I response cytokines and chemokines are associated with the disease (Lindqvist et al. 2013; Schröder et al. 2018). Recently, a fascinating study performed scRNA sequencing of the CSF of 11 patients with PD and cognitive impairment, versus 11 sex and age matched controls, together with immunohistology staining of the mid brain. They found significantly more T cells in the substantia nigra, which were close to  $\alpha$ -Synuclein deposits in Tyrosine Hydroxylase (TH) neurons and partially displayed a CD4 Th17 phenotype. Analysis of the scRNA sequencing revealed clonal expansion of CD8 T cells which expressed high levels of CD44 and IL-7R $\alpha$ . Among the clonally expanded CD4 T cells they found an upregulation of CXCR4 and other genes involved in tissue residency (*CD69*, *Junb*, *Jak1*) in PD patients. Additionally, Th17 cells were found adjacent to dopaminergic neurons (Gate et al. 2021).

Recently, a study has shed light on the presence of CD4 and CD8 T cells in the brain. Galiano-Landeira et al. reported the presence of significantly more CD8 T cells, but not CD4 T cells, in the brain parenchyma of PD cases. They further observed that the density of CD8 T cells positively correlated with the loss of dopaminergic neurons. Besides this, those CD8 T cells expressed cytotoxic molecules (Granzyme A, B and K), markers of tissue residency and IFN $\gamma$ . Studying the number of CD8 T cells in a Lewy Body disease case in early stages, they found a robust T cell infiltration preceding neuronal loss and  $\alpha$ -Synuclein aggregation. As the CD8 T cells were in close proximity to the neurons, the authors discussed that CD8 T cells might be the initiators of the neuronal loss (Galiano-Landeira et al. 2020).

Mouse models have served to gain deeper insight into the role of the adaptive immune system in disease processes. Two mouse models are mainly used, one of them by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and the other by injection of human  $\alpha$ -Synuclein expressed by an adeno-associated virus (AAV2), which causes a specific loss of dopaminergic neurons in the substantia nigra. In the MPTP model, it was shown that T cell-deficient mice had greatly reduced dopaminergic neuron degeneration, implying that this drug-induced model involves the adaptive immune system (Brochard et al. 2008). In line with this, another team demonstrated that in the MPTP model, Fingolimod (Sphingosine 1-phosphate surrogate that induces T cell retention in SLOs) treatment led to a restoration of dopaminergic neuron counts (Williams et al. 2021). The authors suggested that Fingolimod could be used as a disease modifying treatment option for PD patients. Fingolimod is used for the treatment of MS and could be proposed in other inflammatory disorders (Pournajaf et al. 2022). To my knowledge, Fingolimod has not yet been applied for treatment of PD patients.

IFN $\gamma$ -signaling seems to be important in PD disease. A group in Spain demonstrated that chronic Parkinsonian macaques had higher levels of IFN $\gamma$  and TNF than asymptomatic macaques (Barcia et al. 2012). In IFN $\gamma$  deficient mice treated with MPTP, a strong reduction in PD symptoms was observed. The authors argue that IFN $\gamma$  plays a role in microglia and astrocyte activation. Also in the brain parenchyma of PD patients, more than 60% of the CD8 T cells are positive for IFN $\gamma$  staining in the cytoplasm (Galiano-Landeira et al. 2020). A recent study showed that in a mouse model of AAV2 induced PD, T cells infiltrating the brain produced high levels of IFN $\gamma$ , pointing towards a crucial role of type I response involved in the loss of dopaminergic neurons in the mid brain (Williams et al. 2021). Interestingly, Churchill et al. treated MPTP-intoxicated mice with Copaxone, which increased the Th2/Th1 ratio and Treg cells, and showed partial restoration of dopaminergic neurons (Churchill et al. 2019). Both mouse models converge towards a role of IFN $\gamma$ -signaling in PD progression, which can be treated by Copaxone. This could act as an interesting treatment option for PD patients as this drug is already clinical approved for the treatment of MS (Bioko et al 2012).

The above findings were summarized in a hypothetic model illustrating the potential auto-immune contribution of T cells to dopaminergic neuron loss (**Fig. 1.15**). Further studies are necessary to assess how T cells are involved in PD disease progression and clinical trials are needed to test whether T cell targeted drugs (Natalizumab, Fingolimod, Copaxone) could potentially serve as disease-modifying treatments for PD patients.



**Figure 1.15 Hypothetical model of T cell involvement in Parkinson's disease.** Activated T cells reactive to  $\alpha$ -Synuclein or other neuronal antigens differentiate into effector CD8 T cells or Th1/Th17 cells and penetrate the brain through the blood-brain barrier, guided by the CXCR4-CCL12 axis. Once in the CNS, CD4 T cells interact with microglia and astrocytes, which leads to the activation of the latter and the production of neurotoxic molecules and cytokines. Treg cells play a neuroprotective role in suppressing hyper activation of microglia and astrocytes. The secretion of CCL2 may lead to recruitment of further leukocytes, importantly phagocytes and CD8 T cells. Whether CD8 T cells are involved in the disease course is currently unclear and supported by only few studies showing clonal expansion of CD8 T cells in the CSF and CD8 T cells with a  $T_{RM}$  phenotype in the brain parenchyma of PD patients.

#### 1.2.4 Alzheimer's disease

Alzheimer's disease (AD) currently affects 44 Million people worldwide. People with AD suffer from dementia including symptoms of disorientation and cognitive impairment such as memory loss and delusions. This results in loss of confidence for affected patients in daily activities and finally leads to death 8-10 years after disease onset. This disease is associated with elderly people, as the disease risk from the age of 70 years doubles every 5 years (Lane et al. 2018). The pathophysiology of the disease includes the accumulation of beta amyloid peptides ( $A\beta$ ) plaques and neurofibrillary tangles (NFTs) consisting of hyper phosphorylated tau proteins, which are neurotoxic. These  $A\beta$  peptides proceed from the amyloid precursor protein (AAP), which is consequently cleaved in the CNS by  $\beta$ -secretases such as BACE-1 and the  $\gamma$ -secretases to form extracellular  $A\beta$  aggregates (Soria Lopez et al. 2019).  $A\beta$  peptide aggregates lead to the formation of NFTs, which are associated with advanced stages of AD.

A hallmark of AD patients is the loss of synapses and synaptic plasticity, which leads to cognitive decline. This goes along with inflammatory signs of the CNS including mild T cell infiltration, microglial activation, astrogliosis and neuronal loss (Scheltens et al. 2021). It is widely accepted that myeloid cells play an important role in the disease progression, but the role of T cells is yet to be understood (Dai et al. 2021).

Itagaki et al. discovered in the brain parenchyma of 10 AD patients' T cells a prevalence of CD8 over CD4 T cells (Itagaki et al. 1998). Subsequently, another study reported that T cells in the brain parenchyma displayed a memory phenotype by expression of CD45RO and other markers such as LFA-1 and CD27, correlating with migratory properties of these T cells in the brain parenchyma (Togo et al. 2002). Furthermore, the work of Merlini et al. demonstrated that CD8 T cells can mostly be found in the brain parenchyma of AD patients compared to healthy controls, and most abundantly in the Hippocampus. In this region, the number of T cells correlated most strongly with NFTs, suggesting that CD8 T cells accumulate in advanced stage of AD (Merlini et al. 2016 and 2018).

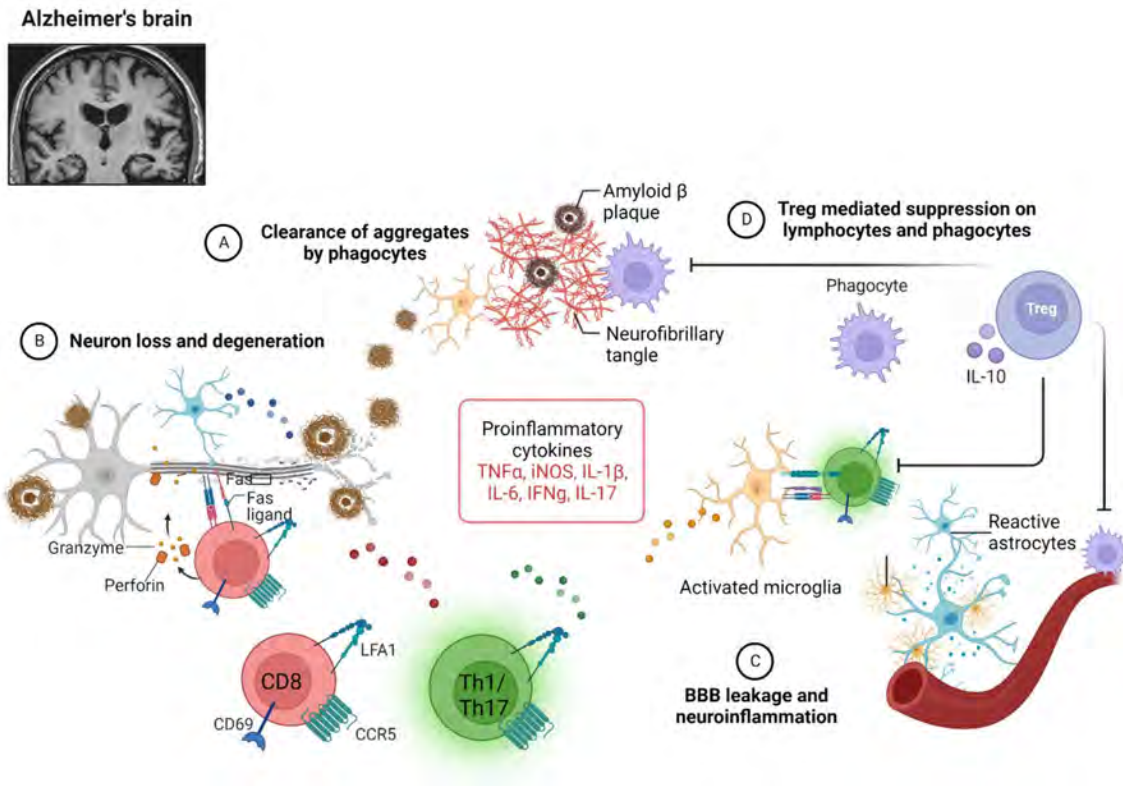
Other studies highlighted the increase of T cells in the CSF of AD patients (Liu et al. 2018). A recent study highlighted the increase of CD8 T<sub>EMRA</sub> cells in the blood of AD patients that correlated significantly with a decline in the cognitive score (Gate et al. 2020). Furthermore, significantly more CD8 T<sub>EMRA</sub> cells were detected in the CSF of AD patients showing high expression of cytotoxic molecules (*GrzA*, *GrzB*, *NGK7*), and chemokines (*Ccl4*, *Ccl5*) - and clonal expansion, suggesting that they have likely recognized an antigen within the CNS. They also found significantly more GrzA expressing CD8 T cells in the brain parenchyma of AD patients that are close to A $\beta$  plaques. This might imply direct elimination of neurons by CD8 T cell-mediated cytotoxic molecules such as granzymes and Perforin in AD disease progression.

In the blood of AD patients, a higher proportion of A $\beta$ -specific T cells are detected (although T cells from healthy controls also respond to A $\beta$  peptides) which express several activation and migration markers upon activation. One study showed that A $\beta$ -specific T cells show enhanced expression of activation markers (CD69) and chemokine receptors (CCR2 and CCR5) and produced pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, IFN $\gamma$  and TNF (Pellicanò et al. 2010). Further studies have confirmed that A $\beta$ -specific T cells from AD patients have high levels of expression of CCR5 and MIP-1 $\alpha$ , which were shown to be important for transmigration across the BBB (Man et al. 2007).

A $\beta$ -specific T cells secreting IFN $\gamma$  appear to be important for disease progression in a mouse model of AD: One team demonstrated IFN $\gamma$  to have a pivotal role in microglial activation, A $\beta$  plaque deposition and cognitive dysfunction. Herein a prevalence of IFN $\gamma$ <sup>+</sup> CD8 A $\beta$ -specific T cells infiltrated the hippocampus (Browne et al. 2013). Of note, a recent study highlighted that CD8 T cells were predominantly found in the brain parenchyma of aged and AD transgenic mice (Altendorfer et al. 2022): They characterized their phenotype and found them to express a gene signature of CD8 T<sub>RM</sub> cells. Finally, other T cells such as CD4 and  $\gamma\delta$  T cells can be involved in AD disease progression: Brigas et al. showed that preceding A $\beta$  plaque formation and tau-pathology, IL-17 produced by  $\gamma\delta$  T cells is responsible for short term memory defects and synaptic dysfunctions, which can be restored by administration of IL-17 neutralizing antibodies (Brigas et al. 2021).

The role of Tregs in AD patients is a double-edged sword: The adoptive transfer of Tregs in AD mouse models ameliorated cognitive function by reducing A $\beta$  plaques (Baek et al. 2016). Furthermore, the administration of recombinant IL-2 induced Treg expansion in the brain and resulted in an improvement in AD symptoms (Dansokho et al. 2016; Alves et al. 2017; Faridar et al. 2020). Nevertheless, elevated levels of phosphorylated Tau correlated with an increased frequency of CD25<sup>+</sup>FOXP3<sup>+</sup>CD4 T cells in patients (Oberstein et al. 2018). In line with this, weekly depletion of Tregs in 5xFAD mice resulted in a reduced burden of A $\beta$  plaques in the CNS concordantly with infiltration of CXCR1<sup>+</sup> phagocytes that cleared those (Baruch et al. 2015). This may argue that, during AD, Tregs infiltration in the CNS has not only neuroprotective roles, but that Tregs could suppress phagocytes that clear A $\beta$  plaques, leading to further disease progression.

Taken together, T cells of AD patients express several chemokine receptors that enable them to cross the BBB, chemokines that trigger the infiltration of other mononuclear cells, and cytokines, which can activate microglia and astrocytes. Targeting T cells or their cytokines could be a novel road to developing specific therapeutics for AD. The above findings are summarized in **Figure 1.16**:



**Figure 1.16 Hypothetical model of T cell involvement in Alzheimer's disease progression.** In the CNS of AD patients, activated CD8 T cells, as well as Th1 and Th17, cells are found. The transmigration across the BBB is possibly triggered by LFA-1 and CCR5, and some studies suggest that CD4 and CD8 T cells express markers of tissue residency, such as CD69, in AD patients. CSF CD8 T cells were shown to express several cytotoxic molecules, which might suggest the direct neuronal killing of neurons charged with Aβ peptides. CD4 T cells can interact with microglia and astrocytes resulting in their activation and the production of neurotoxic molecules and cytokines. Besides their neuroprotective role, Treg cells suppress phagocytes from clearing Aβ plaques and NFTs, leading to further disease progression.

### 1.2.5 Rasmussen's Encephalitis

Rasmussen encephalitis is a rare childhood disease with an average onset age of 6 years. Affected children present with epilepsy and progressive cognitive decline. The disease duration is highly variable, but the acute phase with several epileptic attacks has an average duration of 2-2 ½ years and ends in a residual stage with rare occurring epileptic attacks but persistent neurological deficits (Orsini et al. 2020). In the brain, focal seizures occur with neuronal loss and astrogliosis that are only found in one hemisphere. Reasons for this were speculated to be based on viral infections targeting only one specific brain region or overexpression of a potential auto-antigen in one but not the other hemisphere (Pardo et al. 2014).

An understanding of the mechanism involved in the pathogenesis was obtained through the study of brain tissue and CSF samples of patients as well as animal models based on LCMV infection (Wiendl et al. 2021). CD8 T cells are found within the affected hemisphere of RE patients adjacent to neurons that express MHC-I molecules. Additionally, CD8 T cells polarize Granzyme B towards the neurons and several neurons showed Caspase-3 staining as sign for apoptosis (Bien et al. 2002). CD4 T cells populate the leptomeninges and perivascular spaces, whereas CD8 T cells are found in higher frequency in the parenchyma of the brain samples of RE patients. Compared to controls, an upregulation of CCL2, CCL5, CXCL-9, CCL22, CCL23 and IFN $\gamma$  is observed in brain homogenates of RE patients. Interestingly, IFN $\gamma$  and CXCL-9 levels strongly correlate with the time of onset of the disease suggesting a type I response is involved in disease onset (Owens et al. 2013). A follow up study highlighted that CD4,  $\gamma\delta$  T cells and CD8 T cells in the parenchyma express the T<sub>RM</sub> marker CD103 at a higher percentage compared to controls, and the latter were found in patients with up to 8 years of disease duration (Owens et al. 2016).

Recent publications showed that the early phases of the disease involve the formation of microglial nodules in the brain. These microglia down regulate P2RY12 – a homeostatic marker for microglia – and express high levels of IL-1 $\beta$ , IL-18 and Caspase-1 – a sign of inflammasome activation. Later in disease progression, these nodules express T cell attracting chemokines and are rich in CD8 T cells, which themselves express IFN $\gamma$ . Furthermore, neurons express TLR3 and TLR7, which suggests that viral infections might trigger the onset of the disease (Tröscher et al. 2019). Indeed, another study confirmed the presence of several human herpes viruses (EBV, HSV-1, HHV-6) in the brain of RE patients, with CD8 T cells that polarized Granzyme B towards adjacent, infected cells. Additionally, neurons were filled with Granzyme B, which suggest that Granzyme B might be responsible for the CD8 T cell-mediated neuronal damage. The MRI score correlated with viral burden, suggesting a causal relationship (Wang et al. 2022).

An elegant mouse study used a recombinant LCMV (rLCMV) to study the gene expression of infected neurons upon contact with CD8 T cells (Di Liberto et al. 2018). It was demonstrated that IFN $\gamma$  signaling in neurons results in the recruitment of phagocytes in a CCL2 dependant manner, which phagocytize synapses and thereby disconnect the neurons from their network. The STAT-1 activation in neurons was also seen in the CNS of RE patients. This study brought to evidence that CD8 T cells can damage their target cells not only in a direct fashion but also by indirect mechanisms employing phagocytes.

Several studies have aimed to identify a possible autoantigen driving the disease course by sequencing the TCR repertoire. Schwab et al. identified that T cells from the CNS showed clonal expansion of certain V $\beta$ -clones, which were found in the brain among CD8 T cells that were adjacent to neurons and astrocytes polarizing Granzyme B to their target cells (Schwab et al. 2009). Besides this, those CD8 T clonotypes were selectively expanded in the blood, which speaks for the involvement of antigen-specific CD8 T cells in the disease course. The same team further demonstrated, using in-depth sequencing of TCR clonotypes, that in comparison to other pediatric epilepsy cases, RE patients show strong expansion of certain clonotypes in the blood, which are overlapping with clonotypes in the brain. One clonotype identified in RE patients was directed against EBV (Schneider-Hohendorf et al. 2016).

In summary, CD8 T cells are the predominant population in the brain of RE patients and display a T<sub>RM</sub> phenotype. Nevertheless, the autoantigen(s) driving RE remain elusive and may be revealed in subsequent studies by cloning the identified TCR clonotypes in cell lines and screening peptide libraries for antigens that such TCRs recognize. It may be that the response to specific viruses triggers the onset of RE.

In this Ph.D. Thesis we brought evidence that CD8 T<sub>RM</sub> cells are key to driving chronic damage to CNS neurons. Therapeutic strategies to deplete CD8 T<sub>RM</sub> cells could be an interesting novel approach to see how chronic autoimmune diseases of the CNS can be managed by the elimination of the agents that cause long-term neurological deficits, as is seen in RE patients.

#### 1.2.6 Paraneoplastic disease syndromes and other autoimmune encephalitides

Paraneoplastic cerebral disease (PCD) represents a variety of autoimmune-mediated encephalitides, which are caused by cancer occurring outside the nervous system. These stand in association with autoantibodies against antigens that are expressed both by cancer and by neural cells, of which most are neurons (Dalmau et al. 2018). In the last 15 years, several additional autoimmune neurologic syndromes, associated with cancer or not, have been identified, each associated with characteristic autoantibodies. These autoantibodies target either surface molecules (such as neurotransmitter receptors or adhesion molecules) or intracellular antigens. In the former case, the autoantibodies have been shown (or are highly suspected) to be pathogenic. In the latter case, the autoantibodies are witness to an autoimmune process, but the T cells are thought to mediate neural tissue damage.



Comparative studies conducted in the frame of the California Encephalitis Project highlighted that encephalitis caused by anti-N-Methyl-D-aspartate receptor (anti-NMDAR) antibodies is 4-times more frequent than all encephalitides caused by Herpes simplex virus type 1, varicella-zoster virus and WNV together. However, this chapter will focus on PCD associated with autoantibodies against intracellular antigens summarized in **Table 1**, as the involvement of T cells for those PCD cases.

Antibody (alternative names)	Main associated tumor types
Hu (ANNA-1)	Small cell lung cancer in ~75%
Yo (PCA-1)	Breast cancer (~20%) Gynecological cancer (ovary/fallopian tube ~60%)
Ri (ANNA-2)	Breast cancer ~70% and lung cancer ~25%
Ma1 (PNMA1 and 2)	Various cancers: lung/pleural (~30%), testicular, gynecological tract, non-Hodgkin lymphoma, breast cancer, renal cancer, and melanoma
Ma2/Ta (PNMA2 only)	Most commonly testicular germ cells (up to ~70%) or lung tumors (non- Small cell lung cancer)

**Table 1** PCD cases associated with intraneuronal antigens and the causative cancers

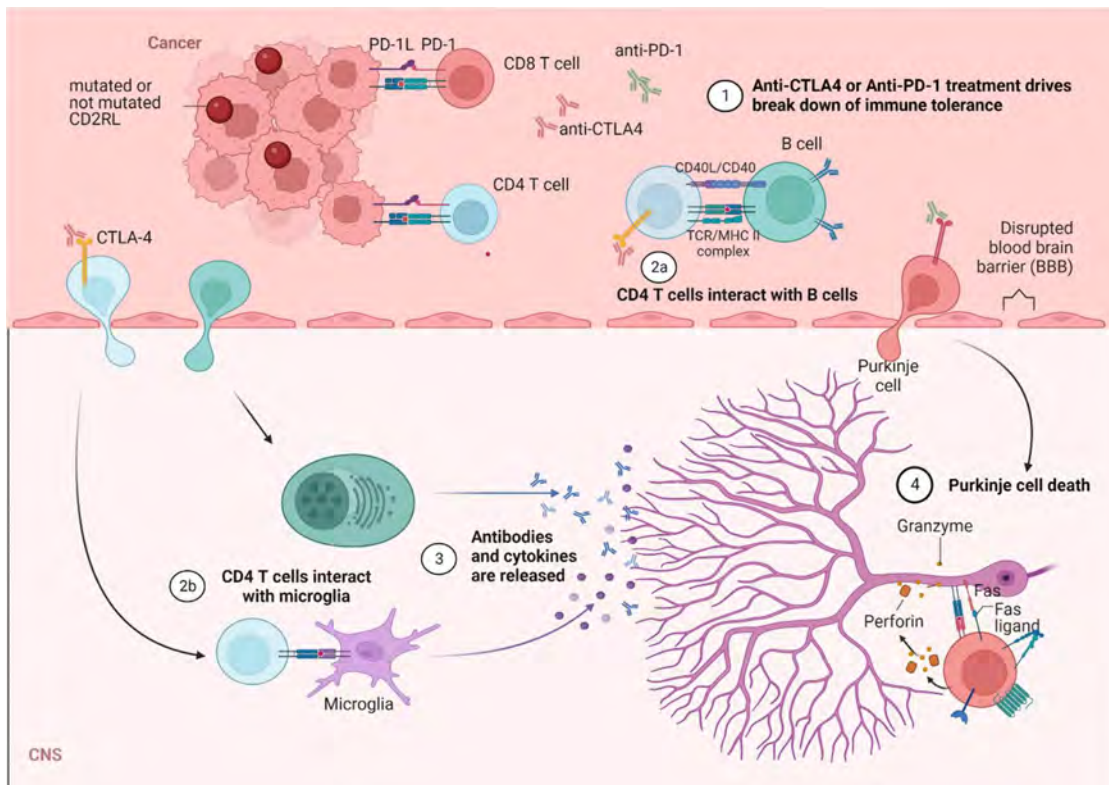
Yo- and Tr-associated antigens are specifically localized in Purkinje cells, which are located in the cerebellar cortex (Rodriguez et al. 1988; Graus et al. 1998). Ri-associated antigens are located in post-mitotic neurons, that are found in the ventral brain stem and are associated with motor function (Buckanovich et al. 1993), while the Hu-associated antigen is an RNA processing protein expressed in a large variety of neurons as it is important for glutamate-mediated neurotransmission (Ince-Dunn et al. 2012).

It is poorly understood why some cancer patients develop PCD syndromes and others do not, since it was shown for anti-Yo cases that the CDR2 and CDR2L antigens are expressed in tumors of patients with and without encephalitis (Raspotnig et al. 2017). Additionally, 7 out of 44 small cell lung cancer individuals without PCD had anti-Hu antibodies in the serum, albeit in lower levels than in encephalitis cases (Dalmau et al. 1990). One possible explanation for this is the genetic pre-disposition of the patients, as it was shown that anti-Yo PCD is associated with the HLA-II haplotype DRB1\*13:01~DQA1\*01:03~DQB1\*06:03 (OR=5.4, p=0.0016), which might suggest that CD4 T cells recognize these antigens with a higher affinity leading to

the breakdown of immune tolerance (Hillary et al. 2018). An important study to address this question found that tumors of anti-Yo patients showed an aberrant infiltration of T and B cells that formed tertiary lymphoid structures within the tumor. Furthermore, the tumor of PCD patients expressed more of the CDR2L variant with gain-of-function mutations leading to antigen over-expression by the tumor (Small et al. 2018). Therefore, both the HLA associations of the patient and the tumor properties could predispose patients to the breakdown of immune tolerance and subsequent cerebral encephalitis (Peter et al. 2022).

In the brain of anti-Yo patients, an infiltration of CD8 T cells was observed in the cerebellar white matter close to Purkinje cells (Giometto et al. 1997). Aye et al. confirmed this finding and characterized Granzyme B-producing cells in the dentate nucleus. An interesting study conducted by Bien et al. aimed to compare the neuropathology in surface receptor and intracellular antigen-mediated PCD. They found an increased infiltration of T cells in the dentate nucleus of the hippocampus, with a higher ratio of CD8 over CD4 T cells in the intracellular antigen group compared to the surface receptor group. Furthermore, these CD8 T cells were located in proximity to neurons and showed polarization of Granzyme B and lytic granules towards neurons associated with a loss of MAP2 neurons in the intracellular antigen group. Conversely, in the surface receptor group, higher staining of C9 was detected on neurons, which suggests that complement-mediated neurotoxicity could be the reason for the neuropathology in the surface receptor group (Bien et al. 2012).

Since the introduction of immune checkpoint inhibitors (ICIs) for cancer immunotherapy, the number of PCD cases has increased, i.e. the number of anti-Ma2 encephalitides increased by 112% after the onset of ICI treatment in France (Vogrig et al. 2019). Although the numbers of PCD cases are rare (i.e. 10/year in France), the administration of ICIs partially triggers the onset of encephalitis within days (Vogrig et al. 2020). This is associated with increased T cell frequency in the CSF, which suggests the role of encephalitic T cells in the disease progression (Gill et al. 2020; Raibagkar et al. 2020). Our team demonstrated a novel mouse PCD model based on the expression of the neo-antigen Hemagglutinin (HA) both at the tumor site and on Purkinje cells, and the adoptive transfer of HA-specific CD4 and CD8 T cells. Importantly, the treatment with anti-CTLA4 (and to a lesser extent anti-PD-1) could elicit Purkinje cell loss. The infiltrating CD8 T cells secreted high-levels of IFN $\gamma$  and TNF in an antigen-specific manner. They were close to Purkinje cells with Granzyme B polarization towards their target (Yshii et al. 2016 and 2019). These findings are summarized in **Fig. 1.17**.



**Figure 1.17 Hypothetical model of paraneoplastic cerebellar disease.** CD4 and CD8 T cells at the tumor site recognize the auto-antigen (i.e. CDR2L) but are restrained in aberrant activation by CTLA-4/CD86 or PD-1/PD-1L interactions. The administration of immune checkpoint inhibitors leads to the breakdown of peripheral immune tolerance, B cell maturation, autoantibody production, and infiltration of T and B cells into the CNS. CD4 T cells further interact with microglia, resulting in the production of neurotoxic molecules and cytokines. Plasmablast can further release autoantibodies. CD8 T cells may directly interact with Purkinje cells and induce apoptosis via the polarization of lytic granules toward the neurons.

### 1.2.7 Toxoplasmosis

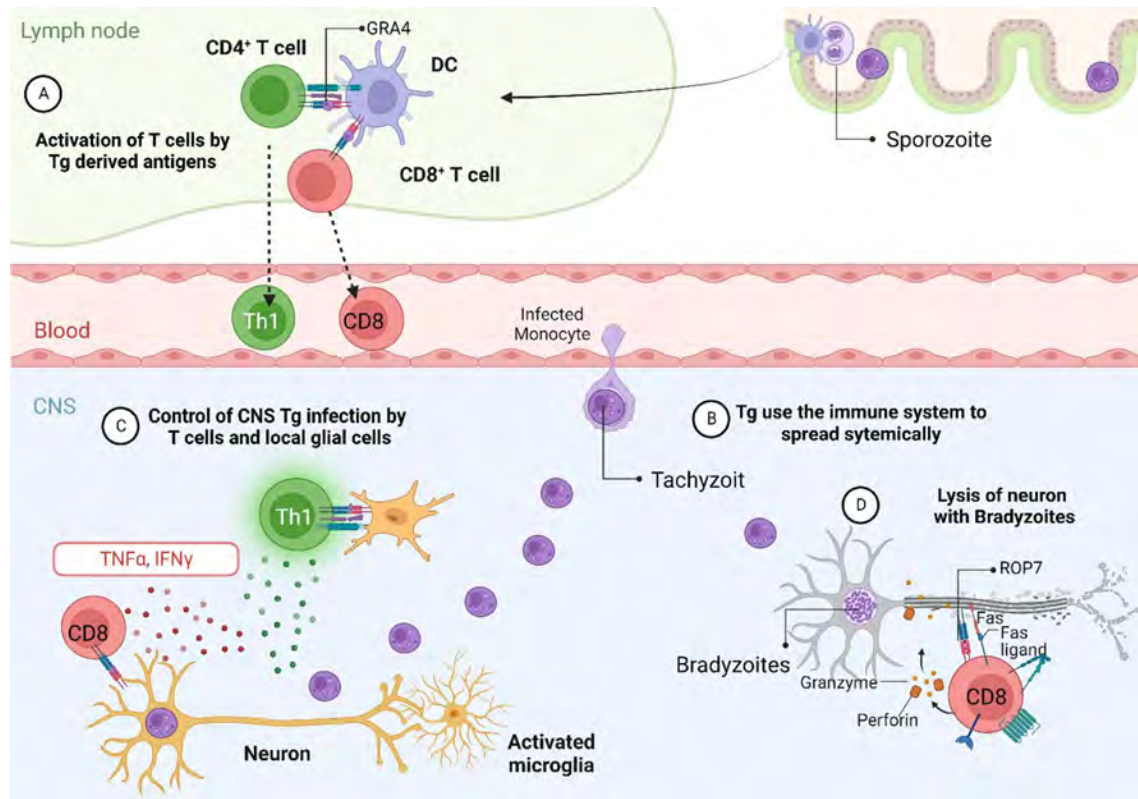
*Toxoplasma gondii* (Tg) is an intracellular protozoan parasite, which definite hosts are felids (i.e., cats). However, it replicates in warm-blood vertebrates as intermediate hosts and it is believed that one-third of the world population is infected by this parasite. The transition is mediated by Sporozoites in the feces of felids, which disseminate in form of Tachyzoites through contaminated food or water into humans (Cerruti et al. 2020). Once taken up in food or water, Tg crosses the intestinal barrier by direct invasion and cell lysis of epithelial cells and spreading into macrophages, dendritic cells, and neutrophils in the lamina propria (Snyder et al. 2021). Tg then disseminates into the body through a lytic cycle in the host immune cells leading to the systemic spreading of the Tachyzoites (Black et al. 2000).

An immunocompetent individual can control the infection, nevertheless, Tg persists in the form of Bradyzoite cysts inside neurons and muscle cells (Remington et al. 1965). Those cysts do not cause pathological damage to the brain, but the Bradyzoites can sporadically differentiate

back into Tachyzoites and cause severe, sometimes lethal, encephalitis when the immune system is not able to control the infection (Frenkel, et al. 1987). This is especially dangerous when Tg infects the fetus during pregnancy. Furthermore, in immunodeficient individuals such as AIDS patients, Tg can spread in the CNS, which is clinically accompanied by headache, fever, and cognitive dysfunction such as disorientation and confusion. If the infection is not controlled, Tg triggers encephalitis, which involves brain lesions consisting of a large number of macrophages and T cells, wherein CD8 T cells play a crucial role (Graham et al. 2021).

Tg is first recognized by DCs, which mature and migrate to mesenteric lymph nodes, where they activate T cells to initiate the adaptive immune response (Poncet et al. 2019). Animal models further supported this hypothesis, as CD4 and CD8 T cell-depleted mice died within two weeks upon Tg infection (Gazzinelli et al. 1991). Additionally, blocking IFN $\gamma$  with a monoclonal antibody resulted in the same lethal course with a large number of Tachyzoites in the brain, causing encephalitis (Suzuki et al. 1988). The study by Guiton et al. pointed out the major role of CD8 T cells, rather than CD4 T cells, in the control of Tachyzoites in the brain (Guiton et al. 2009). In humans, the treatment of a patient with etanercept (soluble TNFR inactivating TNF) resulted in Tg-induced encephalitis (Gonzalez-Vincent et al. 2003). Interestingly, transcripts of T cells and TNF are highly enriched in mice infected with Tg (Gazzinelli et al. 1993).

Mouse models have contributed to the understanding of this CD8 T cell response for the control of Tg in the brain. Kang et al showed that the adoptive transfer of CD8 T cells alone was sufficient to rescue mice from lethal Tg-induced encephalitis. Interestingly, the antigens recognized by T cells varied during Tg infection: In the acute phase (2 weeks after Tg infection), CD8 T cells recognized mainly GRA4 expressed only on Tachyzoites, while in the chronic phase (6-8 weeks after Tg infection) brain CD8 T cells recognized mainly ROP7 expressed on Tachyzoites and Bradyzoites (Frickel et al. 2008). Despite the essential role of IFN $\gamma$  in controlling Tachyzoites spreading, in chronic Tg-infected mice the adoptive transfer of IFN $\gamma$ <sup>-/-</sup> CD8 T cells leads to a significant reduction of Bradyzoite cysts, while that of Perforin<sup>-/-</sup> CD8 T cells was unable to eliminate brain cysts, which suggest that Perforin is an important regulator of cyst persistence in the brain (Suzuki et al. 2010). This mechanism is dependent on the MHC-I presentation of the Tg antigens by the infected neurons to CD8 T cells (Salvioni et al. 2019). These findings are summarized in **Figure 1.18**.



**Figure 1.18 Host control of *Toxoplasma gondii* infection.** Once *Toxoplasma gondii* (Tg) is uptaken by the food in the form of sporozoites, it disseminates in the body importantly via immune cells. Tg is first recognized by DCs, which migrate to lymph nodes and initiate an immune response by activating CD4 and CD8 T cells. The tachyzoites are cleared by IFN $\gamma$ - and TNF-mediated mechanisms, but Tg can persist in neurons in the form of bradyzoite cysts. These cysts can be partially cleared once an infected neuron presents ROP7 peptides to a CD8 T cell, which is able to kill infected neurons through perforin-mediated mechanisms.

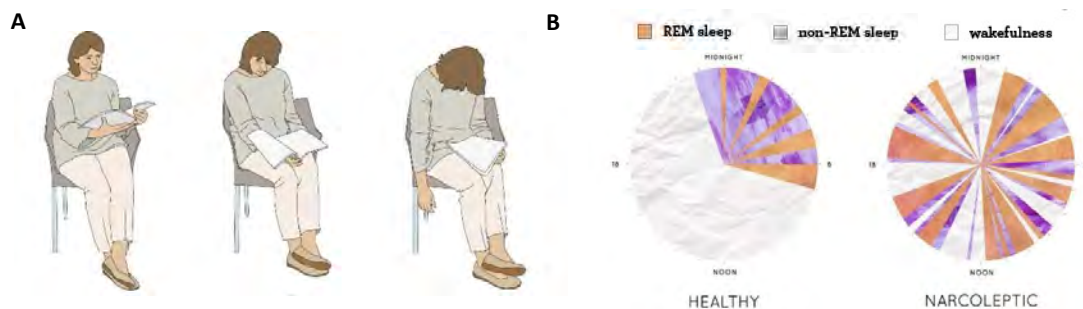
Besides the functional aspect of CD8 T cells in the brain, efficient memory formation is essential for the control of Tg. IL-15<sup>-/-</sup> mice that were treated with anti-IL7 all succumbed to Tg infection over time, whereas non-treated mice survived (Bhadra et al. 2010). This could point towards a role of brain CD8 T<sub>RM</sub> cells in controlling Tg gondii, as we discussed before the essential role of IL-7 and IL-15 for the long-term survival of CD8 T<sub>RM</sub> cells. Indeed, a recent work highlighted that CD8 T<sub>RM</sub> cells are formed in proximity to Tg gondii cysts and produce high levels of IFN $\gamma$  and TNF (Landrith et al. 2017). We began a collaboration with another team that study the functional properties of CD8 T cells in models of chronic Tg infection (*Porte et al. unpublished work*) to expand the findings of this Ph.D. Thesis to infectious settings.

### 1.3 The pathophysiology of Narcolepsy Type I

#### 1.3.1 Clinical symptoms, epidemiology, and causes of NT1

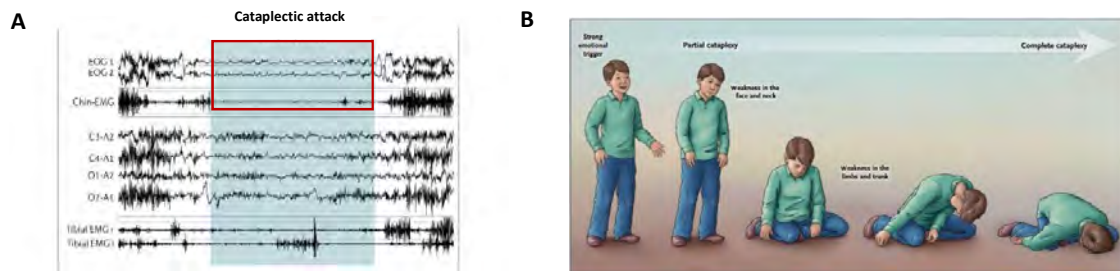
Narcolepsy type 1 (NT1) is the most severe form of central hypersomnia in humans characterized by excessive daytime sleepiness (EDS) and night-time sleep disruption (NSD) (Dauvilliers et al. 2001, Sorensen et al. 2013; **Fig. 1.19A**). During inactive phases, patients fall asleep in an irresistible manner often associated with vivid dreaming due to premature rapid eye movement (REM) sleep. Although the individuals feel for a short moment refreshed after the sleep attack, the feeling of sleepiness soon returns (Dauvilliers et al. 2003). Furthermore, the duration of sleep is short, which leads to a fragmentation of sleep and wake periods during the day rather than one continuous sleep phase during the night (**Fig. 1.19B**).

In many persons with NT1, the sleepiness is severe, resulting in difficulty focusing and staying awake at school and work, thus leading to declining grades at school, poor performance at work, or even motor vehicle accidents (Pizza et al. 2015). Along with vivid dreaming, half of the patients display sleep paralysis at the onset of sleep or at the time of awakening (Dauvilliers et al. 2007). Sleep paralysis is the inability to move the limbs or the head, to speak, or even to breathe normally for a maximum of one minute at awakening. These clinical manifestations led to the development of diagnostic criteria using the Multiple Sleep Latency Test (MSLT), where brain activity, muscle activity, and eye movement are measured while patients take a nap (Carskadon et al. 1986). According to the third international classification of sleep disorders (ICSD-3), NT1 is diagnosed when a person shows a mean sleep latency of fewer than 8 minutes and more than two rapid eye movement periods at sleep onset (SOREMP's; AASM 2014).



**Figure 1.19 Excessive daytime sleepiness and sleep cycles of Narcolepsy Type 1 patients.** (A) A schematic illustration of excessive daytime sleepiness and the inability to stay awake during inactive phases (Bassetti et al. 2019). (B) Typical sleep profile of a healthy (left) or narcoleptic (right) individual showing deep (non-REM in purple) and light (REM in orange) sleep periods over 24 hrs (Wales et al. 2012)

Another important clinical feature to diagnose NT1 is cataplexy, which is a sudden episode of partial or complete paralysis of voluntary muscles triggered by strong, mostly positive, emotions such as laughing (Overeem et al. 2011). The paralysis usually evolves over multiple seconds, first affecting the face (tongue protrusion) and neck and then causing weakness in the trunk and limbs, the person may slump to the ground, fully conscious but immobile for as long as one or two minutes (Scammell et al. 2018; **Fig. 1.20B**). On the electromyogram cataleptic periods are characterized by activity loss in several channels ranging from 2-10 seconds up to more than 2 minutes depending on the intensity of the attack (**Fig. 1.20A**).



**Figure 1.20 Cataplectic attacks in NT1 patients.** (A) An electromyography (EMG) recording during a cataplexy episode (shaded area) documents a loss of muscle tone in multiple channels with superimposed bursts of increased muscle phasic activity (which present clinically as motor phenomena such as muscle twitching and face grimacing). EOG, electro-oculography channel (Bassetti et al. 2019). (B) An illustration of Cataplexy triggered by a positive emotion (in this case laughter; adapted from Scammell et al. 2018).

The onset of the disease usually begins in adolescence between the ages of 10 and 20 years, with 65% of patients developing the disease in the first two decades of life (Dauvilliers et al. 2001). Cases with disease onset after the fourth decade of life are very rare (Dye et al. 2018). NT1 is a lifelong condition, but its diagnosis may take years given the non-specific nature of the initial symptoms (excessive daytime sleepiness). Clear-cut cataplexy is however pathognomonic of NT1.

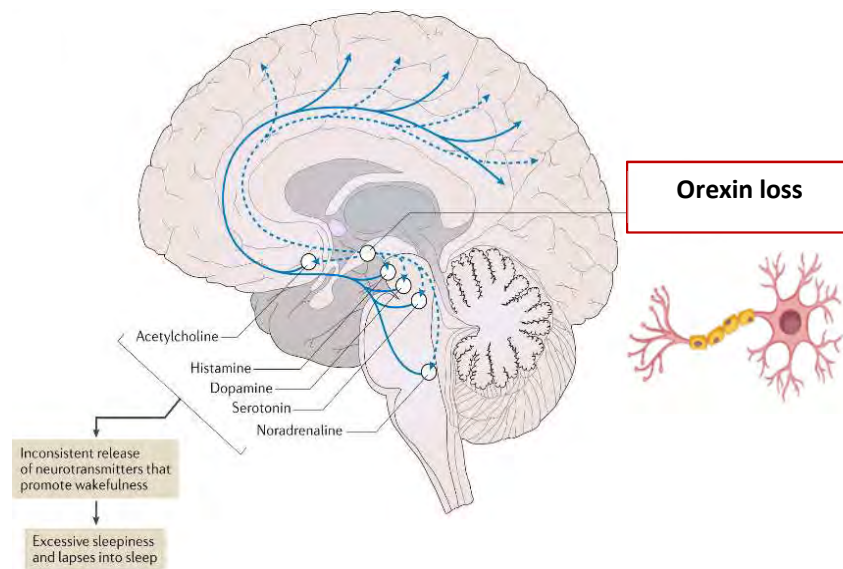
The global prevalence of NT1 is 200-500 per million individuals (Silber et al. 2002). Persons of Jewish ancestry are less (10-40 per million; Wilner et al 1988) and Japanese people are more prone (1'600 per million; Juji et al. 1988) to develop NT1. Children of NT1 patients have a slightly higher risk of developing the disease (1-2% compared to 0.2% in the whole population; Mignot et al. 1998, Hor et al. 2011).

The mechanism underlying NT1 is the deficiency of orexin- (alternative name: hypocretin) signaling. Orexin-A and B are neuropeptides that are produced from the common pre-cursor prepro-orexin (Sakurai et al. 1998, de Lecea et al. 1998). The Orexin 1 or Orexin 2 receptors are widely distributed in the CNS, especially in regions of the hypothalamus, basal forebrain, tuberomammillary nucleus, periaqueductal gray, dorsal raphe, and locus coeruleus, which promote wakefulness, feeding behavior, and locomotion (Trivedi et al. 1998; Chieffi et al. 2017)

A post-mortem analysis of brains revealed that NT1 patients have a reduction of more than 80% in orexin-producing neurons in the tubular hypothalamus (Peyron et al. 2000; Thannickal et al. 2000). Some Orexin-neurons were still present in the brains of patients, indicating that individuals with NT1 were not born without these neurons (Savvidou et al. 2013). Rather the loss of these ~70,000 Orexin-neurons emerges later in life, being closely linked to the onset of symptoms (Moresco et al. 2018; Pizza et al. 2014). This disappearance of the neuronal population is highly specific, as the adjacent melanin-concentrating hormone neurons are not altered in number compared to healthy individuals during the disease course (Peyron et al. 2000; Thannickal et al. 2000). However, a recent study described a profound decrease in corticotropin-releasing hormone neurons in the paraventricular nucleus of NT1 patients, the significance of which remains unknown (Shan et al. 2022).

Orexin neurons were shown in electrophysiological studies to fire most actively during wake-phases and sleep-to-wake transition (Mileykovskiy et al. 2005; Lee et al. 2005). The group of Mileykovskiy could additionally show that these neurons show reduced activity during eating, which is interesting given that NT1 patients tend to develop obesity, and some gain an additional 20% of their body mass index, at disease onset (Kok et al. 2003). Those neurons have wide projections in the olfactory bulb, cerebral cortex, thalamus, hypothalamus, locus coeruleus, dorsal raphe, tuberomammillary nucleus, laterodorsal tegmentum/pedunculopontine nucleus, and the basal forebrain, where they stimulate noradrenergic, serotonergic, histaminergic and dopaminergic neurons that are important for sustaining wakefulness (Barateau et al. 2022 and **Fig. 1.21**). Furthermore, orexin-neurons dampen the activity of  $\gamma$ -aminobutyric acid (GABA)ergic premotor neurons, which, when active during REM periods, inhibit motor neurons leading to muscle atonia (Mahoney, et al. 2019). Therefore, the almost complete loss of these neurons might explain the lack of sustained wakefulness, sleep paralysis and cataplexy observed in NT1 patients.





**Figure 1.21 Neuronal circuits of orexin-neurons and consequences of orexin neuron loss during NT1.** Orexin neurons help sustain wakefulness during the daytime by stimulating a variety of wakefulness-promoting neurons in the cortex, brain stem, and basal forebrain. With the loss of the orexin-producing neurons in NT1 patients, neurons in these target regions fire less consistently, leading to sleepiness and lapses into sleep. Pathways shown in blue lines are excitatory. Open circles indicate neuron-cell bodies, and dotted lines indicate reduced activity when orexin-neurons are lost; adapted from Bassetti et al. 2019

Nowadays, the loss of this neuronal population is appreciated by the orexin levels in the cerebral spinal fluid (CSF) after lumbar puncture. A CSF orexin level lower than 110 pg/ml is now sufficient to diagnose NT1 according to ICSD-3 (AAM 2014). During the disease course, a progressive drop in orexin levels in the CSF was observed in several patients when a second lumbar puncture took place 12 or more months after the initial sampling (Lopez et al. 2017). Besides this, a study including 55 patients with intermediate orexin levels in the CSF (200-110 pg/ml) correlated lower orexin levels with a higher frequency of cataplectic attacks (Postiglione et al. 2022). This observation strongly supports the idea to treat NT1 early on with disease-modifying treatments before the complete and irreversible loss of orexin neurons. Such treatments targeted especially the immune system for reasons detailed in further sections, with the attempt to block the disease course. Nevertheless, until today the underlying mechanisms leading to the destruction of Orexin neurons remain unknown and no disease-modifying treatments of NT1 are currently approved.

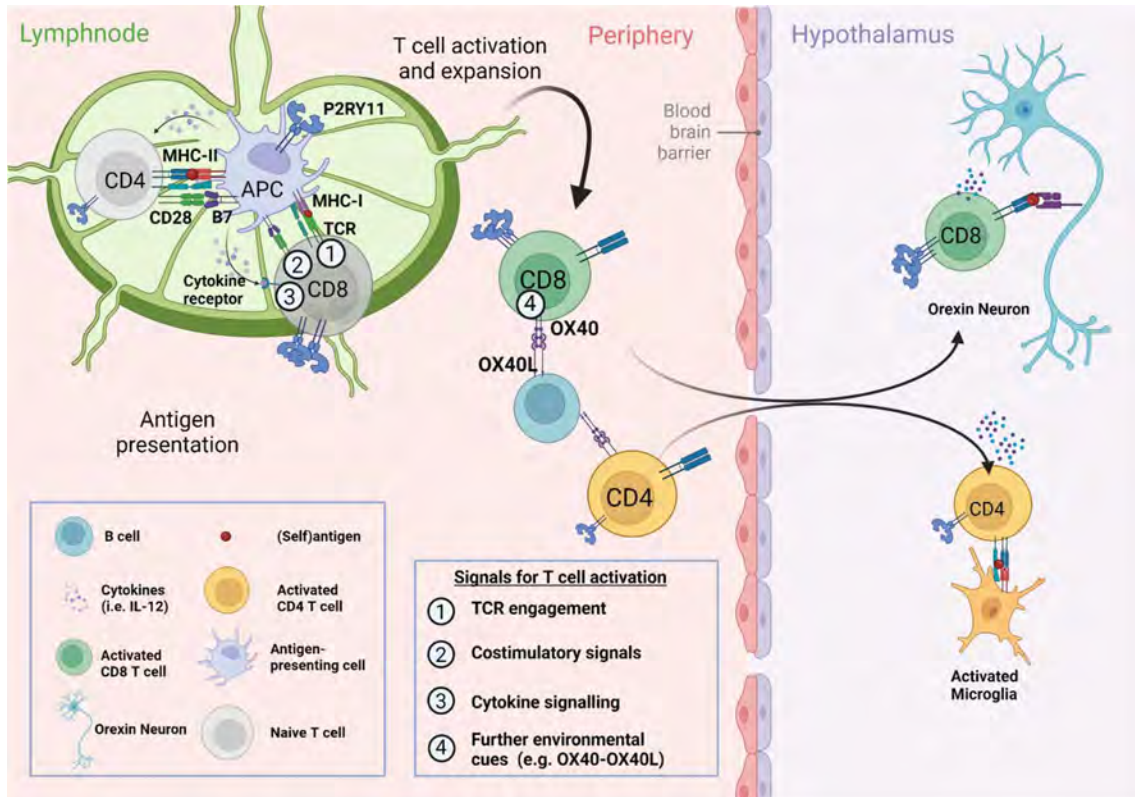
Narcolepsy without cataplexy (and without a decrease of HCRT CSF levels below 110 pg/mL) is referred to as Narcolepsy Type 2 (NT2) and its pathophysiology is even less understood than that of NT1.

### 1.3.2 Genetic immune-related factors in Narcolepsy Type 1 patients

Immune-related genetic factors lead to the autoimmune hypothesis for the development of NT1, including the strong association with the *HLA-DQB1\*06:02* allele of the MHC-II complex. More than 98% of people with NT1 carry this allele, as compared to about 17% of the Caucasian population (providing an odds ratio of >250), a finding that makes the strongest association between an HLA allele and disease known so far (Tafti et al. 2014). Further studies found polymorphisms in the TCR $\alpha$  locus (Hallmayer et al. 2009), which was further confirmed in genome-wide association studies (GWAS) in Europe (Faraco et al. 2013) and China (Han et al. 2013). Regarding these gene loci, one could suggest that the presentation of specific foreign and self-antigens to CD4 T cells might have an important role in the disease course.

These GWAS studies identified further immune-related genes such as Cathepsin H and TNFS4 (OX40L), which play a role in antigen-processing and effector/memory T cell generation through costimulatory signals, respectively. Cathepsin H and OX40L are both highly expressed in antigen-presenting cells such as DCs and B cells. Cathepsin H enzymatic activity was shown to be increased in an autoimmune mouse model of Sjögren's syndrome (Li et al. 2010). OX40-OX40L interaction was shown in mouse models to be essential for the generation of Treg cells, and deficient OX40-OX40L signal leads to the development of a variety of autoimmune diseases, i.e. EAE (Naoto et al. 2010). Mutations in these molecules might therefore pre-dispose individuals to developing auto-immune diseases due to deficient Treg development and possible auto-antigen presentation via Cathepsin H.

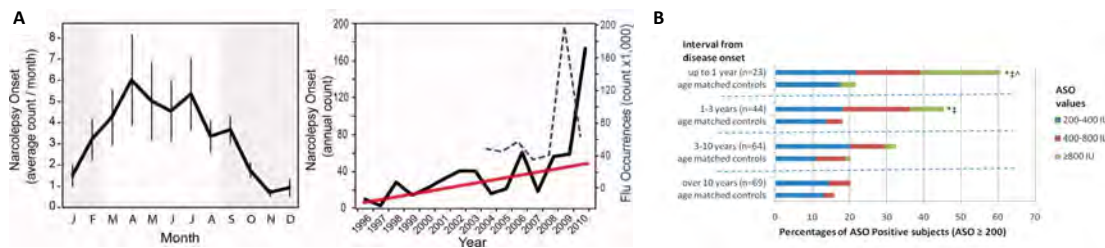
Other polymorphisms in HLA class I loci (Ollila et al. 2015; Tafti et al. 2016) might implicate the involvement of CD8 T cells in the disease progression of NT1. Another GWAS study discovered polymorphisms in the allele for the receptor P2RY11 associated with NT1, which is important for T cell survival (Kornum et al 2011). Further, P2RY11 signaling in DCs is known to be important to release IL-8 and decrease IL-12 secretion. Therefore, a partial loss of function in this receptor might have induced cytotoxic CD8 T cells due to increased IL-12 secretion (Matilda et al. 2017). A recent study also showed a positive correlation between reduced expression of P2RY11 and sleep fragmentation (Rannveig et al. 2021). Besides this, a loss-of-function mutation in the *PRF1* gene (encoding for Perforin) was identified as a protective allele for NT1 development (Ollila et al. 2018). These gene polymorphisms in NT1 are strongly correlated with the immune system, especially T cells, suggesting that T cells are implicated in the disease (**Fig. 1.22**).



**Figure 1.22 Susceptibility genes for NT1 might play a role in antigen presentation and T cell activation.** Schematic illustration of T cell activation in a hypothetical model of pathogenic pathways involved in NT1: During the activation of a naïve T cell, the TCR recognizes its cognate (self)antigen presented by antigen-presenting cells in the lymph node. T cells then leave the lymph node after their initial activation and require further signals to promote their long-term survival and memory differentiation as well as effector function, which are provided by environmental cues presented by other hematopoietic and non- hematopoietic cells.

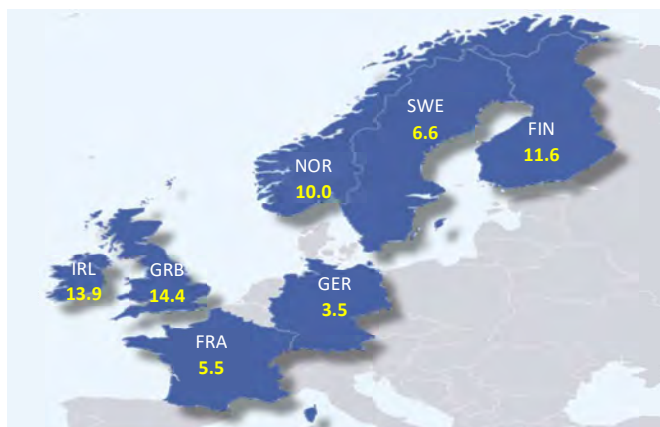
### 1.3.3 Environmental factors associated with Narcolepsy Type 1

NT1 onset seems to occur seasonally in children, according to a study in China which included 906 NT1-diagnosed children from September 1998 to February 2011, with onset being highest in April and lowest in November. This suggests flu infections precede NT1 onset as discussed by the authors (Han et al. 2011). The study further correlated H1N1 infection during the pandemic in 2009/2010 with a higher relative incidence of NT1 children who have been diagnosed since October 2009 when the pandemic began (**Fig 1.23 A**). However, serological studies were not performed to formally ascertain whether the NT1 patients had indeed been recently infected by the flu virus. Another study showed high titers of anti-streptolysin O (ASO) and anti-DNase B (ADB) antibodies in 200 NT1 cases, compared to 200 age-matched controls, suggesting a recent streptococcus infection (Adi et al. 2009; Longstreth et al. 2009). Furthermore, the titer of ASO and ADB antibodies increased when the onset of symptoms was closer (onset within one year), which suggests that streptococcus infection could trigger NT1 onset (**Fig 1.23 B**).



**Figure 1.23** NT1 onset is seasonal and correlates with streptococcus and influenza infection. **(A)** Anti-Streptococcal antibodies in patients with Narcolepsy and age-matched controls (Aran et al. 2009). **(B)** Mean  $\pm$  standard error of the mean of monthly occurrences in the percentage of 12 months across the year (mean of 15-year data). Onsets are approximately 6- to 7-fold more frequent in the late spring/early summer versus late fall/early winter (shaded in gray; see also Table). **(C)** Yearly counts of narcolepsy onset, with a linear trend line (red) depicting modest yearly increases from 1996 to 2009, also projected to 2010. The number of yearly 2004–2010 influenza infections documented by governmental statistics is depicted in blue (Han et al. 2011).

However, one environmental factor especially obtained elevated media attention: The H1N1 influenza virus pandemic in 2009 led to a massive vaccination campaign worldwide. Pandemrix® - the AS03-adjuvanted H1N1 flu vaccine from GlaxoSmithKline - was the most widely used vaccine with more than 31 million vaccinated individuals in 47 countries (Mereckiene et al. 2012; Nguyen et al. 2016). Data from several retrospective studies in Finland, Sweden, Ireland, England, Norway, Germany, and France showed a 3- to 14-fold increase in the relative incidence of narcolepsy in vaccinated children and adolescents (**Fig 1.24**; O’Flanagan et al. 2014; Dauvilliers et al. 2013a; Partinen et al. 2014; Partinen et al. 2012; Wijnans et al. 2013b; Wijnans et al. 2013a). Interestingly, this increase was only observed in HLA-DQB1\*06:02<sup>+</sup> individuals, confirming the importance of this MHC-II molecule to present specific foreign and self-antigens. A study in Finland reported that the increased risk for vaccinated children to develop NT1 was limited to 2 years after the vaccination and the most pre-dominant within the first year (Persson et al. 2014). Nevertheless, the mechanisms underlying this association remain unclear.



**Figure 1.24** Increase in the relative incidence of NT1 in Europe. A map of Europe indicating each country where studies have been conducted the increase in the relative incidence of NT1 in yellow; adopted from Nguyen et al. 2016

The principle of vaccination relies on the ability of our immune system to acquire a “memory” of the vaccine components that originate from the targeted pathogen – yet in an attenuated form that does not cause the disease. Upon encounter with the pathogen, the immune systems elicit a rapid and highly efficient response to clear the pathogen. The memory is carried out by the adaptive immune system consisting of B cells or T cells, which can develop into long-lasting memory cells upon antigen-recognition. Nevertheless, for the development of memory cells, the use of adjuvants is necessary, as the pathogens are inactivated before application to dampen their infectious potential. This results in a weakened signal, which must be overcome by an adequate adjuvant (Di Pasquale et al. 2015; Pellegrino et al. 2015). The described side-effects of vaccines are mild and transient, but recently claims in the medical literature regarding the risk of developing autoimmune diseases as a consequence of vaccination have been accumulating (Guimarães et al. 2015; Karussis and Petrou 2014). Only a small number of these claims are solidly backed by epidemiological studies. The association of NT1 with Pandemrix<sup>®</sup> vaccination is probably one of the most robust associations to date (together with the vaccine-induced immune thrombotic thrombocytopenia side-effect of the adenoviral-encoded SARS-CoV-2 vaccines).

The mechanism that may have led to the onset of NT1 could involve a potential molecular mimicry between H1N1 components in the Pandemrix<sup>®</sup> vaccine and orexin-neuron antigen(s). In line with this, recent studies showed cross-reactive antibodies in Pandemrix<sup>®</sup>-vaccinated individuals between Flu nucleoprotein and Orexin-receptor 2 (Ahmed et al 2015). Another study identified autoreactive CD4 T cells in NT1 patients specific against a C-amidated form of Orexin<sub>54-66</sub> and Orexin<sub>86-97</sub> (Luo et al. 2018). When they analyzed the TCR repertoire of tetramer-positive T cells, they found shared motifs between influenza pHA<sub>273-287</sub> and Orexin<sub>54-66</sub> specific clones with an overlap in the CDR3 $\beta$  TRBV4-2-CASSQETQGRNYGYTF and TRAV2-CAVETDSWGKLQF-TRAJ24 regions, suggesting molecular mimicry. Consistent with these findings, Vuorela et al. reported homology similarity between NA<sub>175-189</sub> and Protein-O-mannosyltransferase 1 (POMT1<sub>675-689</sub>). The authors argued that this could be a clue for molecular mimicry.

Nevertheless, another study cloned the isolated, previously identified TCR clonotypes recognizing flu and self-reactive antigens into Jurkat cells and tested them for their functionality (Luo et al. 2022). No cross-reactivity was able to be confirmed between NP<sub>17-31</sub> or pHA<sub>273-287</sub> and Orexin<sub>54-66</sub> specific TCRs, which does not support the hypothesis of molecular mimicry between Orexin and influenza-derived peptides.

Another possibility is a large, non-specific stimulation of the immune system (“Bystander activation”) through the AS03-adjuvant with increasing brain inflammation and BBB permeability, allowing the autoimmune process to reach the Orexin-neurons and thus resulting in neuron loss (Sarkanen et al. 2018). The effect of the AS03 adjuvant in the global immune activation cannot be neglected, as autoreactive CD4 T cells against Orexin-peptides have even been discovered in healthy patients, which could be enabled to cross the blood-brain-barrier upon application of the vaccine (Lattore et al. 2018). However, another vaccine applied in Canada, Arepanrix<sup>®</sup>, containing the same Adjuvant AS03, did not induce an increase in the relative incidence of NT1 among adolescents (Jacob et al. 2015), which tends to speak against bystander activation being the (sole) mechanism at play.

Another mechanism involves superantigens, which are of bacterial origin such as strains of *Streptococcus*, and can cross-link certain TCR $\beta$  with MHC-II complexes without the need for classical antigen recognition. This leads to broad immune system activation and the production of pro-inflammatory cytokines such as IFN $\gamma$ , TNF, and IL-2 that enable immune cell infiltrations in various organs including the CNS (Commons et al. 2014). One could argue that *Streptococcus* or influenza infection/vaccination may have triggered autoreactive T cells to be activated, enter into the CNS, and mount an autoimmune response against Orexin Neurons. The evidence for an activated immune system in NT1 will be discussed in the following chapter.

#### 1.3.4 Autoimmunity in NT1

NT1 patients display a peculiar immune signature: One study showed an increase in regulatory T cells in the peripheral blood, which displayed an activated phenotype being HLA-DR<sup>+</sup> and expressing GITR (Lecendreux et al. 2017). Besides this, the expression of CD69 was elevated on CD4 T cells and the frequency of CD4 T<sub>CM</sub> cells was increased. This confirmed the study of Hartmann et al. 2016 revealing that CD4 and CD8 T cells expressed CD69 and produce pro-inflammatory cytokines such as IL-2 and TNF (Hartmann et al. 2016). Furthermore, CD4 T cells expressed IL-21, which is a B cell-supporting cytokine, and there was a tendency towards an increased frequency of B cells in the blood of NT1 patients. Another study confirmed that NT1 patients show an increase in the number of follicular helper T (T<sub>FH</sub>) cells and B cells, although T<sub>FH</sub> from NT1 patients showed a reduced capacity to promote survival and differentiation of memory B cells (Nguyen et al. 2018).

Several studies have tried to identify elevated cytokines or chemokines in the blood of NT1 patients, but they either failed or could not repeat previous findings, except for IL-6 which was found to be elevated in two articles (Himmerich et al. 2006; Okun et al. 2004; Tanaka et al.

2014). A possible reason for this is that blood sampling was performed late after disease onset. Another is that changes in the blood serum are minimal, given the very localized immune attack in the hypothalamus.

Therefore, two studies aimed to measure cytokine and chemokine levels in the CSF of NT1 patients. One study identified very few changes (IL-4; Dauvilliers et al. 2014), although most of those samples were collected more than 12 months after disease onset. Kornum et al. chose to include 4 patients with very recent onset (4 days to 1 month) compared to 12 NT1 patients with controls with longer disease onset and 9 patients with other sleep disorders (Kornum et al. 2015). This study revealed an increase in CXCL10 concentration and a tendency for CXCL9, LT $\alpha$ , and CCL5 to be enriched in the CSF of very recent onset cases. Given the enrichment of CXCL10 and a tendency for CXCL9, LT $\alpha$  and CCL5 the authors argued in support of a type I response implicated in the disease onset. From my point of view, the study design with other sleep disorders is optimal to show an NT1-specific immune response. However, as other sleep disorders also show the presence of activated T cells in the CSF (Lippert et al. 2018), measuring cytokine and chemokine levels of healthy individuals would have been an important additional control.

In 2010, a Swiss research group aimed to address the question of whether auto-antibodies are involved in the disease course of NT1. They designed a flagged poly(A)-binding protein cDNA sequence expressed under the control of the Orexin promotor to identify mRNA/proteins preferentially expressed by Orexin Neurons. Autoantibodies against one of those protein identified in mice, TRIB2, was then identified in 20 out of 119 NT1 patients (14%) compared to 5% in controls (Cvetkovic-Lopes et al. 2010). In the same year, two studies identified TRIB2 autoantibodies in NT1 patients in a cohort from America (Kawashima et al. 2010) and Japan (Toyoda et al. 2010). Nevertheless, these findings could not be replicated in subsequent years. This might lead to the consideration that a virus or vaccination circulating this year might have biased the antibody repertoire in the serum (Kornum et al. 2021).

Further autoantibodies were identified in later studies in NT1 patients against NEI/ $\alpha$ MSH (Bergman et al., 2014), the hypocretin receptor 2 (HCRTR2; Ahmed et al., 2015) and prostaglandin receptor D2 (Sadam et al., 2018). Nevertheless, HCRTR2 is not expressed in orexin neurons (Vassali et al. 2015) and other antigens are also not exclusively expressed in Orexin neurons.

This raises the question of whether the presence of such autoantibodies indeed reflects the cause of NT1, or is just a secondary consequence of a viral infection or vaccination. Finally, Tanaka et al. attempted to assess whether the production of anti-TRIB2 antibodies is the cause or the consequence of Orexin neuron destruction (Takara et al. 2017). TRIB-2 immunized rats showed an increase in anti-TRIB2 antibody titers both in the blood and the CSF, but the number of Orexin neurons in the hypothalamus remained unaffected. The authors concluded that anti-TRIB2 antibodies are unlikely to be the cause of NT1.

Given these negative results, several studies elucidated to identify changes in the T cell compartments. The post-mortem analysis of brains from NT1 patients revealed gliosis but no signs of the inflammatory process (Peyron et al. 2000). These studies were performed years after disease onset and could therefore be the result of burnt-out lesions. One case of symptomatic NT1, occurring in the frame of Ma2-antibody-induced encephalitis disease could give access to a brain sample 4 months after NT1 onset. Post-mortem brain analysis showed focalized CD8<sup>+</sup> T cells infiltrating the hypothalamus, together with a loss of Orexin neurons (Dauvilliers et al. 2013b). This raises the question of whether autoreactive T cells, especially CD8 T cells, could be involved in the direct killing of Orexin neurons.

Several recent studies have highlighted the presence of autoreactive CD4 and CD8 T cells against Orexin peptides in the blood. Latorre et al. used T cell libraries to identify Orexin-peptide reactive T cell clones. This is a very sensitive assay, which allows the identification of the presence of autoreactive T cells with very low frequency in the blood. In all 16 patients, autoreactive CD4 T cells against several Orexin peptides were detected, which spanned the whole protein. In contrast, autoreactive CD8 T cells were detected only in 3/10 NT1 and 2/3 NT2 patients. The study highlighted that the antigen-presentation was HLA-DR and not HLA-DQ restricted despite the genetic pre-disposition for the HLA-DQB06:02 allele. Of note, one NT2 patient with normal Orexin-levels in the CSF showed abnormal high frequencies of Orexin-peptide reactive CD4 T cells and CD8 T cell clones and developed NT1 symptoms in the follow-up (Latorre et al. 2018). The authors discussed that these autoreactive T cells might have initiated the loss of Orexin Neurons and the onset of NT1-specific syndromes.

Another study identified autoreactive CD4 T cells against Orexin<sub>86-97</sub> peptides (Jiang et al. 2019). Besides this, they characterized expanded autoreactive CD4 T cell clones and found them to highly express T-bet, IFN $\gamma$ , and Perforin. Another study performed RNA sequencing on POMT1<sub>675-689</sub> specific CD4 T cells from NT1 patients and found them to upregulate the gene expression of *IFN $\gamma$* , *perforin 1*, *Granzyme B*, *CCL1*, *CXCL-9* and *CXCL-10* (Vuorela et al.



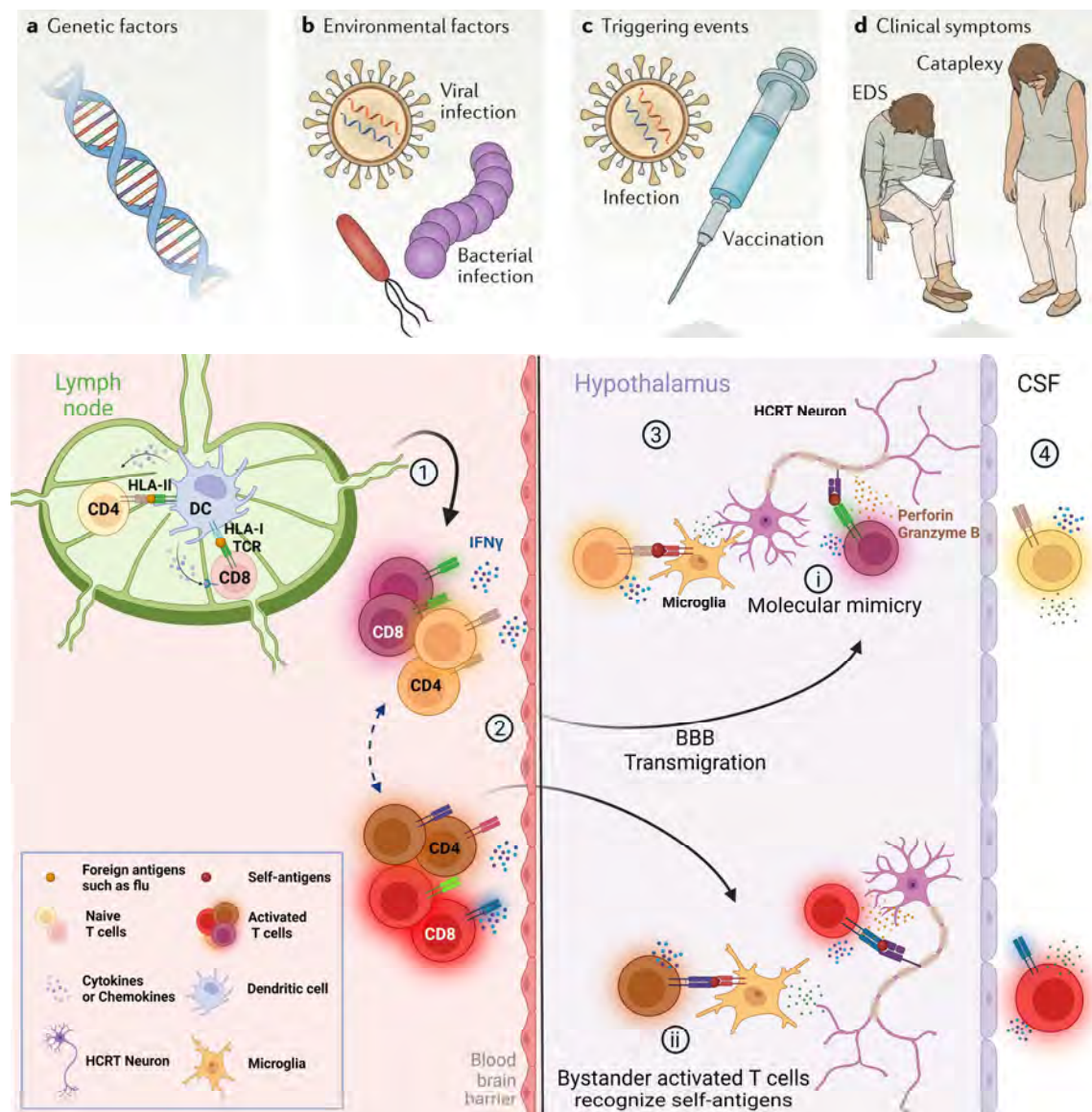
2021). Further studies investigated the cytokine profile of autoreactive CD4 and CD8 T cells from 27 NT1 children, compared to 15 healthy children, and revealed significantly higher levels of IFN $\gamma$  and TNF in the presence of orexin-peptide pools. This difference was not detected in adults (Cogswell et al. 2019).

CD8 T cells were also shown to recognize other NT1-relevant peptides. Surprisingly, albeit no difference in the frequency of CD8 T cells recognizing Orexin peptides was identified, they recognized other brain autoantigens: The recognized peptides were RFX4 and LHX9, which are intracellular proteins produced by neurons including Orexin neurons. Interestingly, 9 out of 9 patients carrying the HLA-I risk alleles HLA-C\*04:01, HLA-B\*51:01, and HLA-C\*11:01 recognized those peptides compared to only 4 out of 11 NT1 without the HLA-I risk allele, which might indicate that the genetic predisposition favors Cd8 T cells recognizing those autoantigens (Pedersen et al. 2019). CD4 T cells reactive against RFX4 were also identified in 4/10 NT1 children (Luo et al. 2021). To sum up, both autoreactive CD4 and CD8 T cells were identified against various peptides from orexin neurons and responded by the production of IFN $\gamma$ , IL-2, and expression of cytotoxic molecules (Perforin and partially Granzyme B).

Besides this, recent studies focused on the identification of NT1-specific phenotypes in the CSF, as the CSF drains the brain and can provide information on the local events during the disease course. One study comparing 14 NT1 patients with a long-duration disease, with sex- and age-matched controls, did not find any changes in classic memory subsets between patients and controls (Moresco et al. 2018). Lippert et al. expanded the analysis of CSF T cells to 43 central hypersomnia disease cases (12 NT1, 7 NT2, and 24 idiopathic hypersomnia) and 24 healthy controls. Importantly they found a positive correlation between the frequency of activated CD4 and CD8 T cells and the worsening of ESS parameters, suggesting that those cells might be either involved in or a consequence of the disease severity (Lippert et al. 2018). A recent study reported one case of a five-year-old boy with an important infiltration of CD8 T cells in the CSF, which was analyzed two-month after disease onset (Moresco et al. 2020). This might point towards a pathogenic role of CD8 T cells at the disease onset. Latorre et al. reported in the CSF of one NT2 patient the presence of an Orexin-reactive CD8 T cell clone that recognized Orexin peptides in an MHC-I-restricted manner (Latorre et al. 2018).

Further in-depth analysis of the CSF TCR repertoire of recent onset cases is needed, to discover clonally expanded CD4 or CD8 T cells that might be important for the disease initiation or progression of NT1.

The above findings were summarised in a model of the Orexin-neuron loss that starts, on a genetically-susceptible host, with an external trigger, such as flu or streptococcus infection, which leads to the transmigration of CD4 T cells and CD8 T cells to the hypothalamus and the subsequent destruction of Orexin neurons. The figure was chosen to show findings that were replicated in several patient cohorts and not to imply molecular mimicry, as this is disputable in the literature (**Fig. 1.25**).



**Figure 1.25 Model of Orexin-Neuron loss in NT1.** (A) The genetics of individual’s favor NT1 development. (B) Environmental factors such as streptococcal or viral infections might predispose individuals to narcolepsy later in life. (C) A triggering event, such as vaccinations and infections, reactivates the immune memory response, leading to a crossing of activated T cells across the blood-brain barrier (D) The clinical symptoms of narcolepsy start and progress from disruption of sleep architecture when more than 80% of orexin neurons are silenced by the immune attack to cataplexy once 95% of orexin neurons are silenced. (E) Schematic representation of the above-mentioned events. Molecular mimicry and bystander activation of autoreactive T cells are depicted as possible mechanisms of the onset of the disease. BBB, Blood-brain barrier, EDS, excessive daytime sleepiness; *adapted from Bassetti et al. 2019*

### 1.3.5 Treatment of Narcolepsy as an autoimmune disorder

Given the peculiar immune signature and since it was hypothesized that NT1 might be an autoimmune disorder, several immune-modulating therapies have been applied to patients. Nevertheless, these treatments were rarely performed in the frame of an appropriate clinical study, with a sufficiently large number of patients with a side-by-side comparison with a placebo group.

Corticosteroids have broad effects on the immune system in dampening the production of pro-inflammatory cytokines and the generation and survival of a large range of immune cells including neutrophils, macrophages, B cells, and T cells (Chatman et al. 2020). Two patients treated with prednisone did not show any improvement in NT1 symptoms (Hecht et al. 2003; Peraita-Adrados et al. 2017), although other studies reported a reduction of cataplectic attacks after prednisone intake in NT1 patients. Nevertheless, the symptoms reappeared after the therapy was stopped, and most importantly the Orexin concentration in the CSF did not normalize, and in some cases even decreased (Coelho et al. 2007; Viste et al. 2017). Given that corticosteroids affect noradrenergic and serotonergic neurotransmitter emission, the above-mentioned observation could also be due to non-immune-related effects (Krugers et al. 2012).

Another treatment with pleiotropic effect is the administration of high doses of intravenous immunoglobulins (IVIg). This pooled antibody agent has several anti-inflammatory effects by depletion of neutrophils and eosinophils, expansion of Treg cell populations, binding to C3a and C5 to suppress the formation of membrane attack complexes and saturating Fc- $\gamma$  receptors to inhibit the action of autoantibodies in immune thrombocytopenic purpura – an antibody-mediated autoimmune disease where this treatment has proven efficacy (Arumugham et al. 2022). Improvements in NT1 symptoms have been recorded in several cases treated with IVIg, including the reduction and severity of cataplectic attacks and the amelioration of EDS. Nevertheless, orexin levels in the CSF remained largely unchanged and symptoms reappeared shortly after the treatment was stopped (Dauvilliers et al. 2006; Dauvilliers et al. 2009; Ruppert et al. 2018). To rule out a possible placebo effect, a small-sized, non-randomized clinical trial treated 22 NT1 patients with IVIG and 30 with a placebo. Placebo and IVIG treatment did not vary significantly in affecting cataplexy or EDS, suggesting that the transient beneficial effect was not related to IVIg administration. Albeit in patients with the most severe symptoms, a slightly significant difference was reported compared to the control group, those patients were pre-selected for the shortest distance to disease onset. Therefore, this biased choice makes this clinical trial difficult to interpret as also discussed by the authors (Lecendreux et al. 2017). One could imagine that the shorter the onset of the disease is, the more patients have hope to be

healed, which – when not controlled by an appropriate placebo control group – can be a simple psychological explanation, that might be independent of the actual biological effect on the immune system.

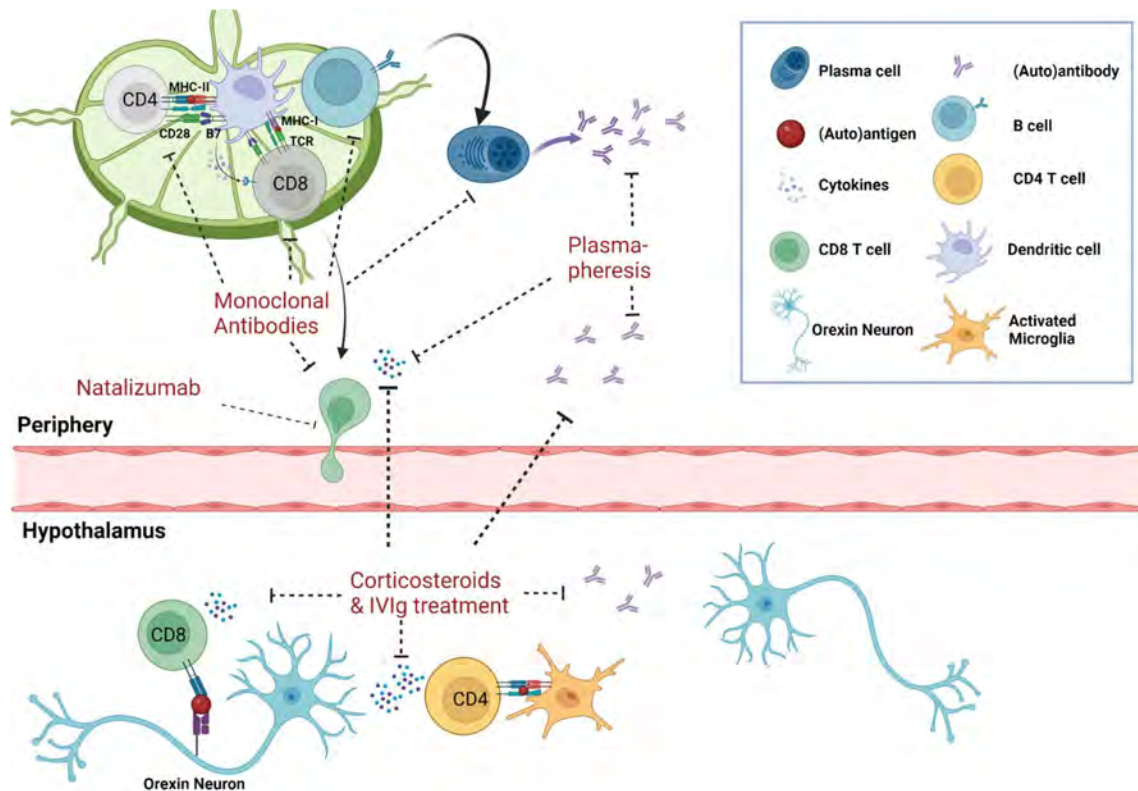
The finding of autoantibodies in NT1 patients led to the treatment of some patients with rituximab, which depletes cells expressing the surface molecule CD20 - most notably B cells but also a population of CD20<sup>dim</sup> T cells. Although the treatment led to a subjective amelioration of EDS in treated patients, the frequency of cataplectic attacks remained the same and the symptoms reappeared (Sarkanen et al. 2016; Wasling et al. 2019).

Another recurrent treatment in autoantibody-mediated pathology is plasmapheresis (PLEX), which removes serum antibodies and other molecules such as cytokines. One patient treated with PLEX two months after disease onset seemed to have an amelioration of NT1 symptoms, nevertheless they reappeared after 3 days (Chen et al. 2005). These negative results and the lack of consistent finding of orexin-specific autoantibodies in NT1 patients speaks against the possibility that autoantibodies might play a pathogenic role in NT1.

In the past decades, new strategies have been applied to broadly target B and T cells with monoclonal antibodies. One 79-year-old narcolepsy patient received alemtuzumab during a mild T cell leukemia (Donjacour et al. 2012). This monoclonal antibody kills cells that express CD52 and leads to sustained depletion of T and B cells. Surprisingly, the patient reported the complete disappearance of his daily cataplectic attacks after treatment with alemtuzumab. Nevertheless, all other symptoms such as EDS remained unchanged for 18 months of treatment. It remains elusive as to why only cataplexy was ameliorated, which could argue for a neuroprotective side effect of alemtuzumab, besides its action on the immune system as discussed in the literature (Jones et al. 2010).

Recently, a 21-year-old woman received Natalizumab treatment, which targets the  $\alpha$ 4- integrin is important for the transmigration of T cells into the CNS. The treatment was started 3-months after the appearance of the first symptoms. The treatment did not affect any NT1 symptoms, even the Orexin level in the CSF dropped from 70 pg/ml to 17 pg/ml within seven months. The treatment might have been started too late in the disease course as the Orexin level was already very low at the time of the Natalizumab application, as discussed by the authors (Scammel et al. 2020).

Those treatments are summarized in **Figure 1.26**.



**Figure 1.26 Treatment of NT1 as an autoimmune disorder.** The most current therapy for treating NT1 so far has been the application of Corticosteroids and intravenous Immunoglobulins (IVIgs) that also exert their role behind the blood-brain barrier as depicted in the graphics; Plasmapheresis targets antibodies and cytokines in the circulation and monoclonal antibodies such as alemtuzumab and rituximab target mainly T and B cell populations, respectively. Natalizumab blocks the transmigration of T cells into the CNS; *adapted from Vringer et al. 2021*

### 1.3.6 Is NT1 an autoimmune disease?

In summary, we evaluate if NT1 is an autoimmune disease according to the following criteria (Damoiseaux et al. 2002; *adapted in* Hayter et al. 2012): i) the specific adaptive immune response is directed to the affected organ or tissue; ii) autoreactive T cells and/or autoantibodies are present in the affected organ or tissue; iii) autoreactive T cells and/or autoantibodies can transfer the disease to healthy individuals or animals; iv) immunization with the autoantigen induces the disease in animal models; v) elimination or suppression of the autoimmune response prevents disease progression or ameliorates the clinical manifestation.

Based on those criteria, MS represents an autoimmune disease: Postulate i) is not fulfilled as the autoantigen is not yet known. Several brain autoantigens have been discovered, which appear more to be the consequence than the initial cause of the disease onset. Nevertheless, recent studies have confirmed postulate ii) as autoantibodies directed against both EBV epitopes and Glial CAM epitopes were identified by sequencing of CSF B cells. The EBV infection stands in close association with the disease onset and could plausibly be an important

initiator of the disease. Indeed, the immunization with the identified Glial CAM peptide induced an MS-like disease in animal models, which fulfills postulate iv). Nevertheless, the transfer of autoantibodies or autoreactive T cells could never trigger the disease onset in humans or animals (postulate iii) is therefore not fulfilled). It has been hypothesized that T cells are the disease initiators and indeed, interfere with T cell migration in early phases of MS patients i.e. Natalizumab administration ameliorates the clinical manifestation, which fulfills postulate v).

In a direct way, for NT1 only the first postulate is fulfilled with the discovery of hypocretin-specific CD4 and CD8 T cells in the blood of NT1 patients. Nevertheless, some indirect evidence for the autoimmune hypothesis of NT1 exists. Further experiments will be discussed, which may either prove or disprove that NT1 is triggered by an autoimmune process:

Regarding postulate ii), the study of Lattore et al. in 2018 shed light on the identification of an orexin-reactive (HCRT<sub>97-124</sub>) CD8 T cell clone in the CSF of an NT2 patient. Another NT2 patient had relatively high frequencies of orexin-reactive CD4 and CD8 T cells in the blood and developed full-blown NT1 several months later. The authors discussed that NT2 patients could develop NT1 symptoms once the destruction of Orexin Neurons reaches more than 80%, as was shown in human post-mortem analysis. Therefore, postulate ii) was confirmed in the context of an NT2 case, but further studies are needed to identify autoreactive T cell clones in the CSF of NT1 patients. These findings would give a strong, convincing point toward the confirmation of NT1 as an autoimmune disorder.

Further, the study of Dauvilliers et al. in 2013 reported a case in which NT1 symptoms developed secondary to an anti-MA-associated diencephalitis. What was striking for this patient is that the presence of CD8 T cells was almost exclusively detected in the hypothalamus, without the detection of a brain tumor. Furthermore, data from our team obtained from an autoimmune mouse model of NT1 support that Orexin-Neuron loss is caused by CD8 T cells, but not CD4 T cells (Bernard-Valnet et al. 2016). Nevertheless, this is indirect evidence for postulate ii), as it is not known which (auto)antigen these CD8 T cells in this secondary NT1 case might have recognized.

Regarding the transfer of autoreactive T cells demanded by postulate iii), one could imagine cloning a functional HCRT-recognizing TCR clone from the study of Luo et al. 2022 and perform *in vitro* killing assays of engineered CD8 T cells together with Orexin neurons obtained by iPSC methods. Our collaborators developed a system to differentiate orexin neurons *in vitro* (Seifinejad et al. 2019), which could be used for such assays.

One study tested the validity of postulate iv) by immunizing mice with TRIB2 autoantibodies. Although TRIB2 auto-antibodies were generated in the mice, they were unable to trigger the Orexin neuron loss. The immunization of humanized mice with orexin-neuron associated autoantigen peptides such as RFX4, LHX9, or the C-amidated form of Orexin<sub>54-66</sub> (expressing those human proteins under the control of the Orexin promotor) would provide more conclusive evidence for the autoimmune origin of NT1. One would need to control the HLA restriction of these peptides to see whether autoreactive T cells are involved. Simplistically, we showed during this Ph.D. Thesis that the immunization of mice with the Pandemrix vaccine causes the destruction of Orexin-Neurons expressing Hemagglutinin (HA), mediated by autoreactive CD4 and CD8 T cells.

For postulate, v) there exists another piece of indirect evidence: A recent study reported the onset of NT1 14 days after the application of the immune checkpoint inhibitor pembrolizumab. The PD-1 inhibitor was applied because the patient suffered from a brain tumor. Strikingly, the woman was 66 years old when she developed full-blown NT1 – an age where the onset of NT1 has very rarely been reported. The CSF analysis showed an infiltration of lymphocytes and a drop in the orexin level. The fact that the breakdown of immune tolerance induced the onset of NT1 is another indirect piece of evidence for the autoimmune hypothesis of NT1. Therefore, a clearer understanding of the immunopathogenesis of NT1 could lead to specific pharmaceuticals that targets the agents driving the disease course.

#### 1.4.1 Novelty and impact of the project

This project aims to obtain new knowledge about the pathogenic immune pathways that may lead to orexin neuron loss in mouse models and, therefore, could shed light on NT1 pathogenesis. With the use of an original mouse model, we hope to overcome major caveats in previous studies of NT1, as patients' blood samples were often taken years after disease onset and post-mortem tissue samples did not show signs of an inflammatory process.

Tissue analysis of ongoing narcolepsy should be much more informative, therefore there is a need to perform studies of NT1 in an appropriate mouse model. To date, mouse models of NT1 have been based on genetic disruption of the orexinergic neurotransmission or the destruction of Orexin-neurons through the expression of a deleterious gene (Tabuchi et al. 2014; Hara et al. 2001). These models could explain the role of the orexinergic system, but they do not allow the study of the process of Orexin-neuron loss. Therefore, our laboratory generated an autoimmune mouse model in which HA from an H1N1 influenza virus strain is selectively expressed in Orexin-neurons (“Orex-HA mice”), which made it possible to promote Orexin-neuron loss by transfer of HA-specific effector CD8 T cells (Bernard-Valnet et al. 2016).

To understand the mechanism leading to the destruction of Orexin-neurons, we made use of a method allowing profiling of the translational-(translated mRNA) changes in a specific cell type during an inflammatory process (“Ribotag-method”; Sanz et al. 2009). Previous work exploiting this method showed that in an infectious setting, neuronal damage was mediated by macrophage-induced synaptic stripping of infected neurons (Di Liberto et al. 2018). These studies had not yet been performed in the context of an autoimmune setting, as we did during my thesis project.

We identified in our autoimmune model the presence of an autoantigen specific CD8 T<sub>RM</sub> cell population, that was key to drive chronic CNS damage. Due to accumulating evidence on T<sub>RM</sub> cells in several CNS autoimmune diseases, there is a need for an understanding of their heterogeneity, persistence, and pathogenic mechanisms in their contribution towards chronic tissue damage. The depletion of T<sub>RM</sub> cells could open the door for novel immune-modulating treatments being applicable far beyond NT1 for several chronic auto-immune diseases.

Furthermore, although recent studies provided more insight into the differentiation signals for CD8 T<sub>RM</sub> cells and how they are established in the tissue by signals from the microenvironment, less is known about the signals that are important for the persistence of CD8 T<sub>RM</sub> cells. T<sub>RM</sub> cells can survive indefinitely in epithelial tissues by changing their cell metabolism (Pan et al. 2017; Hayward et al. 2020; Frizzel et al. 2020).



However, these studies used infectious settings, in which the antigen is cleared. Are other and novel mechanisms involved in the long-term persistence of T<sub>RM</sub> cells in autoimmune diseases, in which the antigen is constantly present? At least for RE patients, we know that T<sub>RM</sub> cells are found up to 8 years after the disease onset and can be sustained in the tissue for a long duration.

This Ph.D. Thesis aimed to provide novel insight into the persistence of CD8 T<sub>RM</sub> cells in an auto-immune model of CNS inflammation. Importantly, we discovered the cytokine IFN $\gamma$  to be important for CD8 T<sub>RM</sub> cell maintenance. The abrogation of IFN $\gamma$  signaling by the genetic depletion of IFN $\gamma$ R1 impaired strongly the expression of several T<sub>RM</sub> markers and their persistence in the brain. We may have discovered a novel immune-modulating therapy to inhibit T<sub>RM</sub> cell survival in the brain.

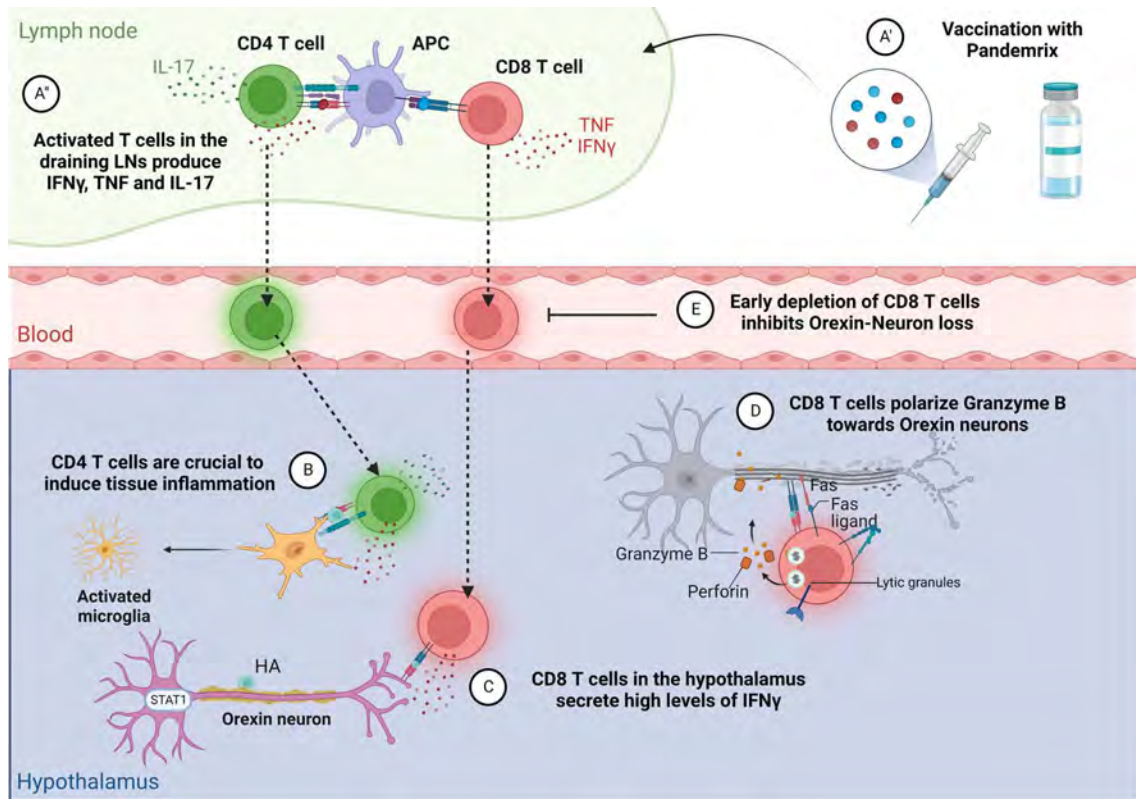
## 2 Results

2.1 Paper: Influenza vaccination induces autoimmunity against orexinergic neurons in a mouse model for narcolepsy.

NT1 represents a neurological disease with a unique disease course and the most severe cause of central hypersomnia in humans. Several retrospective studies revealed an increase in the incidence of NT1 among vaccinated children one to two years after the vaccination with Pandemrix<sup>®</sup>. The underlying cause remains elusive until today.

We developed an autoimmune model of NT1 based on the vaccination of Orex-HA with Pandemrix<sup>®</sup> (containing among other HA of the pandemic H1N1 *influenza virus*) following the transfer of HA-specific naïve CD4 and CD8 T cells. This vaccine-induced NT1 mouse model might be a novel tool to understand how the Pandemrix<sup>®</sup>-vaccination campaign of 2009-2010 lead to an increased incidence of NT1 among children. Through this approach, we can study the effect of the vaccine on “autoreactive” T cells, migration patterns of activated CD4 and CD8 T cells enabling them to cross the blood-brain-barrier, the time course of Orexin-neuron loss, and the pathogenic character of the “autoreactive” T cells in the hypothalamus. Though it has to be kept in mind that findings in this reductionist model need to be validated in patients’ samples to understand how vaccination could have triggered NT1 onset.

With an in-depth understanding of the possible link between vaccination and NT1 gained in this project could help to prevent unwanted side effects of vaccines and once again strengthen society’s trust in vaccination. In the past, we benefited from vaccination to extinguish several life-threatening pathologies. This will be useful in the future when new infections are likely to spread rapidly in a globalized world (i.e. SARS-CoV-2).



## Highlights

- Upon Pandemrix® vaccination, both HA-specific CD4 and CD8 T cells respond by the production of IFN $\gamma$ , TNF, and IL-17 (only CD4 T cells).
- Autoreactive CD4 T cells are **necessary to induce T cell infiltration** in the hypothalamus and subsequent tissue inflammation.
- Autoreactive CD8 T cells in the hypothalamus produce very high levels of IFN $\gamma$ . The injection of **IFN $\gamma$  deficient HA-specific CD8 T cells reduces the loss of orexin neurons**.
- CD8 T cells polarize **Granzyme B** towards orexin neurons.
- CD8 T cells are **indispensable** for the destruction of orexin neurons.



# Influenza vaccination induces autoimmunity against orexinergic neurons in a mouse model for narcolepsy

✉ Raphaël Bernard-Valnet,<sup>1,2,†</sup> David Frieser,<sup>1,†</sup> Xuan-Hung Nguyen,<sup>1,3,†</sup> Leila Khajavi,<sup>1</sup> Clémence Quériault,<sup>1</sup> Sébastien Arthaud,<sup>4</sup> Silvia Melzi,<sup>4</sup> Maxime Fusade-Boyer,<sup>5</sup> Frederick Masson,<sup>1</sup> Matthias Zytnecki,<sup>6</sup> Abdelhadi Saoudi,<sup>1</sup> Yves Dauvilliers,<sup>7</sup> Christelle Peyron,<sup>4</sup> Jan Bauer<sup>8</sup> and ✉ Roland S. Liblau<sup>1,9</sup>

<sup>†</sup>These authors contributed equally to this work.

Narcolepsy with cataplexy or narcolepsy type 1 is a disabling chronic sleep disorder resulting from the destruction of orexinergic neurons in the hypothalamus. The tight association of narcolepsy with HLA-DQB1\*06:02 strongly suggest an autoimmune origin to this disease. Furthermore, converging epidemiological studies have identified an increased incidence for narcolepsy in Europe following Pandemrix<sup>®</sup> vaccination against the 2009–2010 pandemic ‘influenza’ virus strain. The potential immunological link between the Pandemrix<sup>®</sup> vaccination and narcolepsy remains, however, unknown. Deciphering these mechanisms may reveal pathways potentially at play in most cases of narcolepsy. Here, we developed a mouse model allowing to track and study the T-cell response against ‘influenza’ virus haemagglutinin, which was selectively expressed in the orexinergic neurons as a new self-antigen. Pandemrix<sup>®</sup> vaccination in this mouse model resulted in hypothalamic inflammation and selective destruction of orexin-producing neurons. Further investigations on the relative contribution of T-cell subsets in this process revealed that haemagglutinin-specific CD4 T cells were necessary for the development of hypothalamic inflammation, but insufficient for killing orexinergic neurons. Conversely, haemagglutinin-specific CD8 T cells could not initiate inflammation but were the effectors of the destruction of orexinergic neurons. Additional studies revealed pathways potentially involved in the disease process. Notably, the interferon- $\gamma$  pathway was proven essential, as interferon- $\gamma$ -deficient CD8 T cells were unable to elicit the loss of orexinergic neurons.

Our work demonstrates that an immunopathological process mimicking narcolepsy can be elicited by immune cross-reactivity between a vaccine antigen and a neuronal self-antigen. This process relies on a synergy between autoreactive CD4 and CD8 T cells for disease development. This work furthers our understanding of the mechanisms and pathways potentially involved in the development of a neurological side effect due to a vaccine and, likely, to narcolepsy in general.

- 1 Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), University of Toulouse, CNRS, INSERM, UPS, Toulouse, France
- 2 Service of Neurology, Clinical Neurosciences Department, Lausanne University Hospital and Lausanne University, Lausanne, Switzerland
- 3 Vinmec Institute of Applied Science and Regenerative Medicine, Vinmec Healthcare System, Hanoi, Vietnam
- 4 INSERM U1028, CNRS UMR 5292, Center for Research in Neuroscience, University of Lyon 1, Bron, France
- 5 IHAP, UMR1225, Université de Toulouse, INRAe, ENVT, Toulouse, France
- 6 Unité de Mathématiques et Informatique Appliquées, INRAE, Castanet-Tolosan, France
- 7 National Reference Center for Orphan Diseases, Narcolepsy, Idiopathic hypersomnia and Kleine-Levin Syndrome, Department of Neurology, Gui-de-Chauliac Hospital, CHU de Montpellier, INSERM U1061, Montpellier, France
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8 Center for Brain Research, Medical University of Vienna, Vienna, Austria

9 Department of Immunology, Toulouse University Hospitals, Toulouse, France

Correspondence to: Prof. Roland Liblau  
Toulouse Institute for infectious and inflammatory diseases  
CHU Purpan BP 3028, 31024 Toulouse cedex 03  
Toulouse, France  
E-mail: roland.liblau@inserm.fr

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**Abbreviations:** HA = ‘influenza’ haemagglutinin; HLA = human leukocyte antigen; IFN- $\gamma$  = interferon-gamma; MCH = melanin concentrating hormone; NT1 = narcolepsy type 1; TCR = T-cell receptor

## Introduction

Narcolepsy type 1 (NT1), is a rare, chronic and disabling neurological disease, usually starting at adolescence. It is characterized by excessive daytime sleepiness, cataplexy (sudden loss of muscle tone triggered by emotions) and fragmented nocturnal sleep.<sup>1,2</sup> Post-mortem pathological studies revealed that NT1 results from a profound and selective loss of a small population of neurons in the lateral hypothalamus characterized by the production of the orexin/hypocretin neuropeptides.<sup>3,4</sup> To date, there is no curative treatment for NT1, and patients usually require life-long symptomatic therapies.

The aetiology of NT1 is multifactorial, with disease risk determined both by genetics and environmental factors. Although NT1 pathophysiology remains to be deciphered, converging evidence supports an immune-mediated basis. Indeed, the disease is strongly associated worldwide with the human leukocyte antigen (HLA) class II haplotype *HLA-DQA1\*01:02-HLA-DQB1\*06:02* (odds ratio of 250) and weaker associations have been identified with HLA class I alleles.<sup>5–7</sup> Moreover, NT1 is associated with polymorphisms in other immune-related genes, most notably T-cell receptor (TCR)- $\alpha$  and TCR- $\beta$  chains, perforin and cathepsin H.<sup>5–10</sup> The nature of the genes involved in disease susceptibility suggests the importance of both CD4 and CD8 T cells in the disease process. Additionally, an increased incidence of NT1 in children and adolescents was associated with the 2009–2010 H1N1 ‘influenza’ vaccination campaign using Pandemrix<sup>®</sup> in several European countries.<sup>11–14</sup> A reassessment of the risk of narcolepsy in children in England after receipt of the AS03-adjuvanted H1N1 pandemic vaccine concluded to a 7-fold elevated risk of onset of narcolepsy after Pandemrix<sup>®</sup>, which was confined to the first year after vaccination.<sup>14</sup> Interestingly, a recent report suggests that treatment with immune checkpoint inhibitors could cause narcolepsy in genetically predisposed persons.<sup>15</sup> Thus, antigen-specific as well as more general immune stimulation have been associated with development of narcolepsy.

The striking association with HLA and with a specific vaccine, together with the selective nature of the neuronal destruction observed in NT1 strongly supports an autoimmune basis. So far, efforts to identify pathogenic autoantibodies have not been successful. Recent data, however, support the involvement of autoreactive T cells in NT1 pathogenesis. Our mouse model of immune-mediated NT1 provided clear proof-of-principle that a CD8 T cell-mediated autoimmune process could lead to an NT1-like phenotype, associating orexinergic neuron death, sleep attacks and cataplexy episodes.<sup>16</sup> Accordingly, NT1 patients showed a higher frequency of CD8 T cells recognizing NT1-related candidate

proteins, identified using HLA:peptide multimers, when compared to HLA-DQ-matched controls.<sup>17</sup> Moreover, orexin-specific CD8 T-cell clones were detected in the CSF of one patient.<sup>18</sup> In addition, increased frequencies of CD4 T cells of the Th1 phenotype recognizing orexin peptides have been identified in the blood of patients with NT1 compared to *HLA-DQB1\*06:02*-matched healthy controls.<sup>18,19</sup> Single-cell TCR sequencing suggested clonal expansion and molecular mimicry between orexin and ‘influenza’ virus peptides.<sup>19</sup> Overall, these findings collectively point to the involvement of both CD4 and CD8 in the pathogenesis of NT1, in line with the increased propensity of blood CD4 and CD8 T cells to produce B cell-supporting cytokines in NT1 patients as compared to matched controls.<sup>20</sup> However, the interplay between CD4 and CD8 T cells that elicits hypothalamic tissue damage is completely unknown. In addition, it remains unclear whether and how potentially pathogenic autoreactive T cells can be activated by exposure to the Pandemrix<sup>®</sup> vaccine.

In this study, we leveraged our mouse model expressing ‘influenza’ haemagglutinin (HA) selectively in orexinergic hypothalamic neurons and TCR-transgenic mouse lines in which a large proportion of either CD4 or CD8 T cells recognize HA, to identify whether the Pandemrix<sup>®</sup> vaccine could elicit hypothalamic inflammation and loss of orexin-producing neurons and tease apart the mechanisms involved. Using histology, cellular immunology, transcriptomic of sorted T-cell populations, and loss-of-function experiments we investigated the potential contribution of CD4 and CD8 T-cell subsets in the pathogenic processes. The data indicate that autoreactive CD4 T cells are essential to promote hypothalamic inflammation but do not, by themselves, induce loss of orexin-producing neurons. Conversely, autoreactive cytotoxic CD8 T cells are necessary for the selective destruction of orexin-producing neurons.

## Materials and methods

### Mouse strains

The (BALB/cJ x C57Bl/6J)F1 Orex-HA mice are a double transgenic strain expressing HA of an H1N1 influenza virus (A/Puerto Rico/8/34/Mount Sinai) mainly in Orexin neurons<sup>16</sup>. The TCR-transgenic mice (BALB/cJ x C57Bl/6J)F1 6.5-TCR and CL4-TCR, expressing an I-E<sup>d</sup>-restricted HA<sub>110–119</sub>-specific TCR and a K<sup>d</sup>-restricted HA<sub>512–520</sub>-specific TCR, respectively, have been previously described.<sup>21,22</sup> The TCR-transgenic mice harbor the CD45.1 allotype, whereas the recipient mice only express CD45.2. All mice were kept in pathogen-free conditions

within the UMS006 animal facility, Toulouse, France. All animal experiments were performed in accordance with the European Union guidelines following approval of the local ethics committee (APAFIS#770-2017010511127939V4).

### Naïve T-cell transfer and mouse vaccination

Naïve HA-specific CD4 and CD8 T cells were purified from 6.5-TCR and CL4-TCR mice, respectively. Spleens and lymph nodes were collected, and the relevant T-cell subset was purified (Dynabeads Untouched Mouse CD4 or CD8 Cells kit; Invitrogen). CD4 T cells were then incubated with anti-CD25 (clone PC61) monoclonal antibody (mAb) for regulatory T-cell depletion. Naïve CD4 or CD8 T cells were further purified based on expression of CD62L (Miltenyi Biotech). Naïve CD4 and/or CD8 T cells ( $5 \times 10^6$  each) were injected intravenously into the recipient mice. For *in vivo* proliferation experiments, T cells were stained before transfer with either CFSE or CellTrace Violet (Invitrogen).

Twenty-four hours after transfer of HA-specific T cells, Orex-HA mice and their littermate counterparts were injected in the gluteal muscle with 50  $\mu$ l of either PBS, AS03 adjuvant or commercial Pandemrix® (kindly provided by GlaxoSmith Kline) containing split virion, inactivated ‘influenza’ A/California/07/2009 (H1N1).

In some experiments, depleting anti-CD8 mAbs (YTS 169.4; 200  $\mu$ g/injection) or control rat IgG2b from BioXCell InvivoMab were injected every 5 days from D5 onwards until the end of the experiment.

### Isolation of hypothalamus-infiltrating mononuclear cells

Mice were deeply anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused intracardially with PBS. Brains were immediately removed, and a hypothalamic block was dissected out, homogenized, and digested with collagenase D (1 mg/ml) and DNase I (10 mg/ml). Hypothalamus-infiltrating mononuclear cells were then collected using Percoll density separation.

### Fluorescence activated cell sorting analysis

Cells were harvested from hypothalamus, spleen, draining lymph nodes (i.e. inguinal and sciatic lymph nodes) and cervical lymph nodes at different time points after vaccination. Prior to staining, cells were incubated with anti-CD16 (Fc $\gamma$ R-Blocker; 4GG2: in-house production). Cells were then stained for cell surface markers and, in some experiments, intracellular molecules. Intracellular cytokine staining was performed following 4 h stimulation with PMA (50 ng/ml, Sigma-Aldrich) and ionomycin (50 ng/ml, Sigma-Aldrich) in the presence of Golgi-plug (0.4% v/v, BD Biosciences). Cell suspensions were stained by using a viability dye and anti-CD11b (M1/70, eBioscience), anti-Thy1.2 (53-2.1, eBioscience), anti-MHC-class II (M5/114.15.2, eBioscience), anti-FoxP3 (FJK-16s, eBioscience), anti-granzyme B (NGZB, eBioscience), anti-CD4 (RM4-5, BD Biosciences), anti-CD8 $\alpha$  (53-6.7, BD Biosciences), anti-CD45.2 (104, BD Biosciences), anti-CD45.1 (A20, BD Biosciences), anti-CD44 (IM7, BD Biosciences), anti-CD62L (MEL14, BD Biosciences), anti-CD11c (N418, BioLegend), anti-Ly6C (HK1.4, BioLegend), anti-CD8 $\alpha$  (YTS156.7.7, BioLegend), anti-IL17A (TC11-18H10, BD Biosciences), anti-IFN $\gamma$  (XMG1.2, BD Biosciences), anti-GM-CSF (MP1-22E9, BD Biosciences), anti-TNF $\alpha$  (MP6-XT22, BD Biosciences) monoclonal antibodies. Data were collected on LSRII Fortessa (Becton Dickinson) and analysed with FlowJo software (Tree Star). The t-distributed stochastic neighbour embedding plots were done using CytoBank (Cytobank Inc.).

### Peptide-restimulation of HA-specific T cells in hypothalamic cell suspensions.

Mice were anaesthetized and perfused intracardially with PBS. Brains were removed and a hypothalamic block was dissected out, homogenized and digested in HBSS medium with collagenase D (1 mg/ml), TLCK (30  $\mu$ g/ml) and DNase I (20  $\mu$ g/ml), all from Roche® Diagnostics. After digestion the hypothalamic cell suspension was resuspended in Percoll (30%; GE Healthcare) and spun at a centrifugation step of 1590g for 30 min without break. Myelin and adipose debris were aspirated, and the cells collected. Hypothalamic immune cell suspensions were stimulated with HA<sub>512–520</sub>-peptide and HA<sub>110–119</sub>-peptide (Genecust; 100  $\mu$ g/ml each) in the presence of Golgi-stop (0.07% v/v, BD Biosciences).

### Haemagglutination inhibition assay

Mouse sera from vaccinated and PBS- or AS03-injected control mice were tested for anti-‘influenza’ antibodies using two viral strains: A/Puerto Rico/8/1934(H1N1) (PR8) and A/Michigan/45/2015(H1N1), kind gift of Dr Richard Webby. Haemagglutination inhibition assays (HAI) were performed as previously described<sup>23</sup> using 1% chicken red blood cells. The HAI titres were determined as the reciprocal dilution of the last well that contained non-agglutinated red blood cells.

### Histopathology

Tissues were fixed in 4% paraformaldehyde, conserved in 70% ethanol, and embedded in paraffin. Immunohistochemical and Immunofluorescence staining of 5- $\mu$ m thick coronal sections was performed as described elsewhere<sup>24</sup> using the following primary antibodies: rabbit anti-orexin A (Phoenix Pharmaceuticals, #H-003-30), rabbit anti-CD3 (SP7; Neomarkers), rabbit anti-CD8 $\alpha$  (Abcam, ab209775), rabbit anti-Iba-1 (Wako, #019-19741), rabbit anti-phospho-Stat1 (Cell Signaling, #9167) and anti-granzyme B (ab4059, AbCam). All secondary antibodies were from Jackson ImmunoResearch Laboratories. Fluorescent preparations afterwards were stained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma), embedded and examined using a confocal laser scan microscope (Leica SP5, Leica) equipped with lasers for 504, 488, 543 and 633 nm excitation. Scanning for DAPI (504 nm), Cy2 (488 nm), Cy3 (543 nm) and Cy5 (660 nm) was performed sequentially to rule out fluorescence bleed-through. For  $\beta$ 2-microglobulin, or-oxin, CD8 co-staining, orexin A staining was first performed on untreated sections followed by biotinylated donkey-anti-rabbit secondary antibody. This was followed by consecutive incubation with avidin-peroxidase and biotinylated tyramide. Next the sections were pre-treated in EDTA 8.5 for 1 h in a household cooking device (Braun). Afterwards, rabbit-anti-CD8 $\alpha$  and goat-anti- $\beta$ 2-microglobulin (M-20, Santa Cruz) were incubated for 1 h at RT. The staining was completed by shred incubation with Cy3-conjugated donkey-anti-rabbit, Cy2-conjugated donkey-anti-goat and Cy5-conjugated streptavidin. Image acquisition was performed with 3DHistec Panoramic 250 slide scanner (3DHistec Ltd.).

For neuronal enumeration, brains were fixed in 4% paraformaldehyde and 30- $\mu$ m thick coronal free-floating sections performed at cryostat. Immunohistochemical detection of orexin+ and melanin-concentrating hormone (MCH)+ neurons was performed by using the antibodies described above. Neuron enumeration was performed on 1/12th of the entire hypothalamus (seven sections of 30  $\mu$ m thickness per brain) by using Mercator software (Explora Nova). Alternatively, 10 hypothalamic sections (5  $\mu$ m) per animal

were stained for orexin by immunohistochemistry. Image acquisition was performed with 3DHistec Panoramic 250 slide scanner (3DHistec Ltd.). Enumeration was performed using the automatic count tool with CaseViewer software (3DHistec Ltd.).

### RNA sequencing of T-cell populations

Orex-HA mice were immunized intramuscularly with Pandemrix<sup>®</sup> vaccine 1 day after the adoptive transfer of naïve (CD45.1<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup>) HA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. At Day 14 post-vaccination, endogenous (CD45.1<sup>-</sup>) and transferred (CD45.1<sup>+</sup>) populations of CD44<sup>high</sup>CD62L<sup>low</sup>CD4<sup>+</sup> and CD44<sup>high</sup>CD62L<sup>low</sup>CD8<sup>+</sup> T cells were isolated from spleens, cervical lymph nodes and hypothalami by fluorescence activated cell sorting in four independent experiments involving 20 to 30 mice each.

Total RNA was extracted from the sorted cells using RNeasy Micro Kit (Qiagen). RNA quality and quantity was checked by Eukaryote Total RNA Pico assay (Agilent) and validated with a RIN >7. Library preparation and RNA-sequencing were performed by the Genomics Facility Basel in Zurich using an Illumina HiSeq 2500, generating 75-bp single-end reads.

Sequences were quality checked using FastQC (version V0.11.2) and FastqScreen (version V0.11.4) prior to aligning to the *Mus musculus* primary genome sequence (Gencode: GRCm38.p5, release M16) using STAR (version V2.4.0i). FastQC, featureCounts (V1.4.5-p1) and PicardTools (version V2.17.3) were used to assess the mapping quality. RSEM (V1.3.0) was used to generate the expression matrix that was then analyzed with DESeq2 (R version V3.4.4, BioConductor version V3.6) to identify the differentially expressed genes (DEGs). ClusterProfiler (R package) was used to classify the rendered DEGs into KEGG pathways, gene ontology (GO) terms, and gene set enrichment analysis (GSEA). These classifications were further interrogated to yield a gene list of interest based on immunological relevance.

Raw fastQ reads were used to extract TCR  $\alpha$  and  $\beta$  sequences with MiTCR and to generate chord diagrams.<sup>25</sup>

### Polysomnographic analyses

These combined EEG and EMG recordings were performed as described.<sup>16</sup>

### Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

## Results

### *In vivo* activation of HA-specific T cells in Pandemrix<sup>®</sup>-vaccinated mice

Our general objective was to investigate whether the Pandemrix<sup>®</sup> vaccine could elicit features of NT1 through molecular mimicry and to decipher the underlying immune pathways. To this end, we made use of our original mouse model, the so-called Orex-HA mice, in which the H1N1 'influenza' virus HA is expressed specifically in orexinergic neurons.<sup>16</sup> To trace the HA-specific T cells following Pandemrix<sup>®</sup> vaccination, the T-cell repertoire of vaccinated mice was spiked with genetically marked (CD45.1<sup>+</sup>) naïve CD4 and CD8 T cells specific for HA, originating from 6.5-TCR and CL4-TCR transgenic mice, respectively (Supplementary Fig. 1A). As a result, on average  $0.87 \pm 0.1\%$  of CD4 T cells and  $0.75 \pm 0.05\%$  of CD8 T cells in the spleen

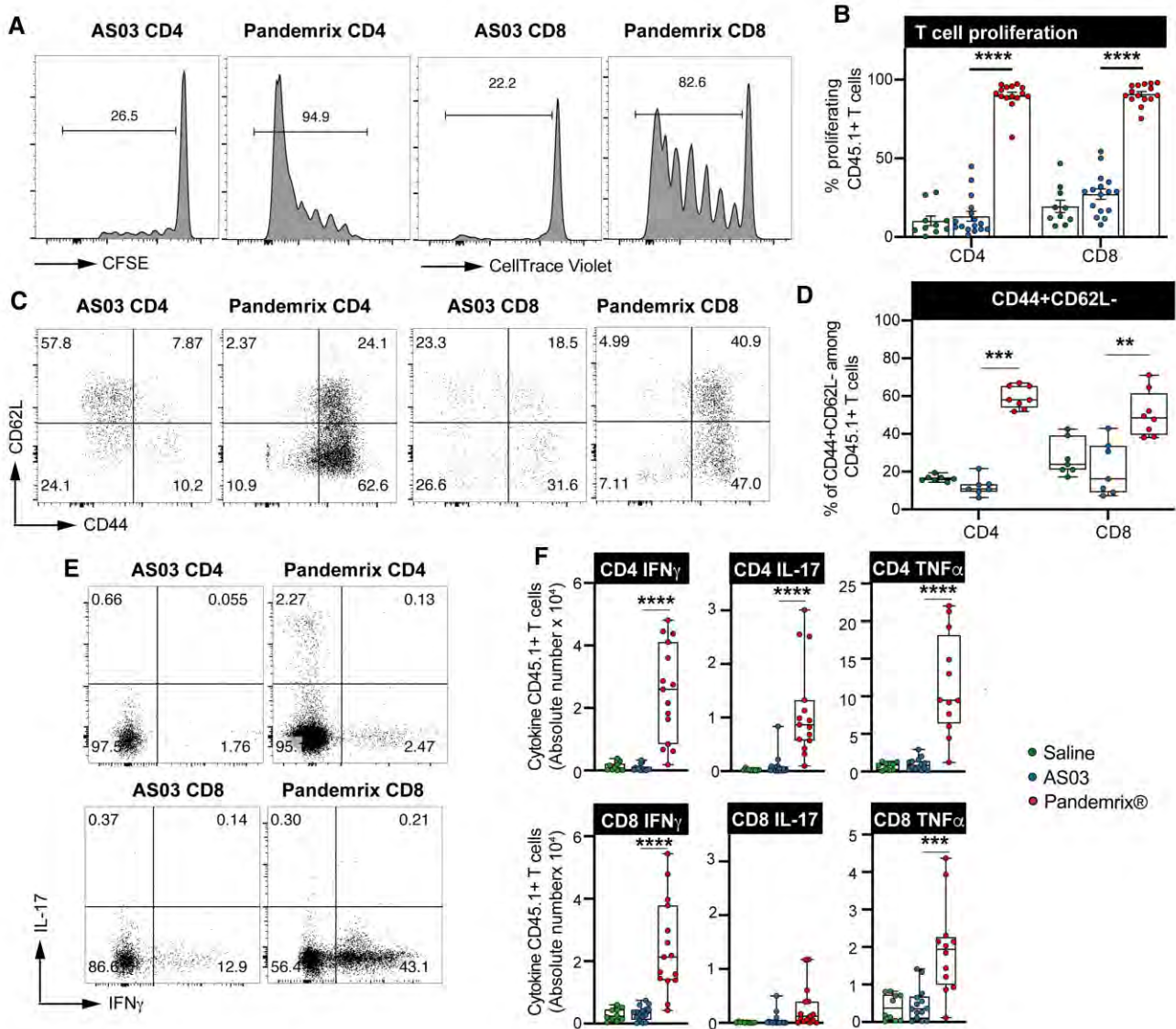
of Orex-HA mice were of transferred origin (Supplementary Fig. 1B). Importantly, the I-E<sup>d</sup>-restricted 6.5-TCR and the K<sup>d</sup>-restricted CL4-TCR recognize HA peptides that are fully shared by the HA subtypes used in Pandemrix<sup>®</sup> and as a transgene in Orex-HA mice (Supplementary Fig. 1B).

To assess whether the human Pandemrix<sup>®</sup> vaccine could activate and expand *in vivo* the naïve CD45.1<sup>+</sup> HA-specific CD4 and CD8 T cells, T cells were stained with a proliferation dye prior to their transfer in either Orex-HA mice or their littermate controls not expressing HA. Recipient mice were immunized intramuscularly the next day with the Pandemrix<sup>®</sup> vaccine, its AS03 adjuvant or saline. Extensive proliferation of both HA-specific CD4 and CD8 T cells was detected following Pandemrix<sup>®</sup> vaccination, but neither with PBS nor with the adjuvant alone, in the draining lymph nodes (Fig. 1A and B) and spleen (data not shown) of both Orex-HA mice (Fig. 1A and B) and control animals not expressing HA (Supplementary Fig. 2A). The *in vivo* Pandemrix<sup>®</sup>-primed HA-specific T cells acquired an activated phenotype, as illustrated by up-regulation of CD44 and loss of CD62L expression (Fig. 1C and D), and effector functions (Fig. 1E and F). Indeed, the transferred HA-specific CD8 T cells differentiated in IFN $\gamma$ - and TNF $\alpha$ -producing effectors, whereas transferred HA-specific CD4 T cells acquired either a Th1 or Th17 phenotype (Fig. 1E and F). Similar results were observed following vaccination of control animals not expressing HA (Supplementary Fig. 2B). By contrast, when used alone, AS03 had a modest effect, if any, on the activation and differentiation of HA-specific T cells in both types of recipient animals (Fig. 1B, D, F and Supplementary Fig. 2A and B). Of note, all tested Pandemrix<sup>®</sup>-vaccinated Orex-HA mice ( $n=14$ ) had anti-HA antibody titres  $\geq 5120$  UI/ml using a pandemic-like H1N1 virus strain, whereas levels in mice injected with the AS03 adjuvant or PBS ( $n=6$ ) were  $<10$  UI/ml. Interestingly, detection of anti-HA antibodies was fully negative in serum of 14 Pandemrix<sup>®</sup>-vaccinated Orex-HA mice when using the PR8/34 H1N1 strain, suggesting lack of cross-reactivity with the HA expressed in neurons.

### HA-specific T cells induce selective destruction of orexin<sup>+</sup> neurons in vaccinated Orex-HA mice

We next assessed, by immunohistochemistry, whether the vaccine-induced activation of HA-specific CD4 and CD8 T cells resulted in CNS inflammation, as a consequence of HA expression in orexinergic neurons. Two weeks after CD4 and CD8 T-cell transfer and Pandemrix<sup>®</sup> vaccination, T-cell infiltration was observed in the hypothalamus in all Orex-HA mice and in none of the non-transgenic controls (Fig. 2A and B). Importantly, T-cell infiltration was minimal or absent in the other investigated brain regions, such as thalamus, hippocampus and cortex (Supplementary Fig. 3A and B).

This finding led us to further characterize, using flow cytometry, the immune cell types accumulating in the CNS of Orex-HA mice. Contrasting with control mice treated in parallel, the CNS of Orex-HA mice harboured large numbers of CD4 and CD8 T cells 2 weeks after vaccination (Fig. 2C and D). The infiltrated T-cell population was dominantly composed of CD8 T cells [mean and range; 54% (35–69)], including both transferred [16% (4–41)] and endogenous CD8 T cells [38% (18–65)]. Transferred CD4 T cells were rare [5% (1–15)] while endogenous CD4 T cells represented 45% (30–64) of CNS-infiltrating T cells (Fig. 2D). Because infiltrated T cells preferentially localized in the hypothalamus of vaccinated Orex-HA mice, we evaluated the kinetics and composition of the T-cell infiltration selectively on dissected hypothalamus. The hypothalamic T-cell infiltration was detected as soon as 7 days post-vaccination, peaked



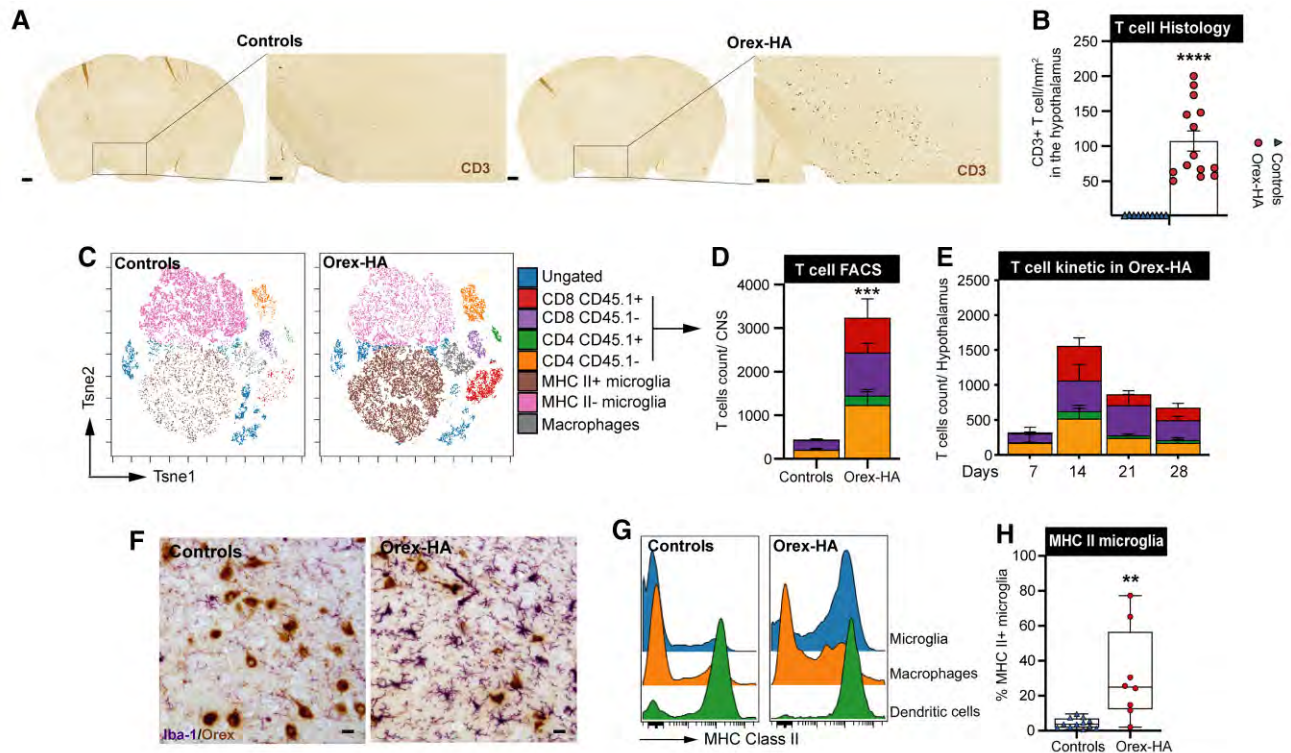
**Figure 1** Activation of HA-specific T cells in the draining lymph nodes upon Pandemrix® immunization of Orex-HA mice. (A and B) Evaluation of the *in vivo* proliferation of transferred (CD45.1+) naïve HA-specific CD4 and CD8 T cells of Orex-HA recipient animals 5 days after intramuscular immunization with Pandemrix® (red circles), the AS03 adjuvant (blue circles) or saline (green circles). (A) Representative fluorescence activated cell sorting (FACS) plot of CFSE or Celltrace Violet dilution in CD45.1+ CD4 or CD8 T cells, respectively, and (B) histograms. Results are expressed as mean  $\pm$  SEM of 10 to 16 mice per group from six independent experiments. (C) Representative FACS plots of CD44 and CD62L expression on CD45.1+ HA-specific CD4 and CD8 T cells 5 days after intramuscular immunization with Pandemrix® or the AS03 adjuvant. (D) Frequencies of CD45.1+ HA-specific CD4 and CD8 T cells harbouring the CD44+CD62L- activated phenotype. Box plots depict interquartile range and median, and whiskers extend between minimum and maximum values of seven to eight mice per group from two independent experiments. (E) Representative FACS plots of IL-17 and IFN $\gamma$  expression in CD45.1+ HA-specific CD4 and CD8 T cells 5 days after *i.m.* immunization with Pandemrix® or the AS03 adjuvant. (F) Absolute numbers of CD45.1+ HA-specific CD4 (top) and CD8 (bottom) T cells producing IFN $\gamma$ , IL-17, TNF $\alpha$  5 days after injection of either PBS, AS03 or Pandemrix®. Box plots depict interquartile range and median, and whiskers extend between minimum and maximum values of 10 to 16 mice per group from six independent experiments. Statistical analyses were performed using the Mann-Whitney test. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

at Day 14 (on average  $1562 \pm 467$  T cells per hypothalamus) and persisted for at least 4 weeks (Fig. 2E). From Day 14 onward, CD8 T cells of transferred or endogenous origin dominated the hypothalamic infiltrates (Fig. 2E). The hypothalamus of control mice at Day 14 contained on average 104 T cells. In addition, a large population of CD11b<sup>+</sup> MHC class II-expressing myeloid cells was also present in the CNS of vaccinated Orex-HA mice, composed of CD11b<sup>+</sup> CD45<sup>dim</sup> microglia and CD11b<sup>+</sup> CD45<sup>hi</sup> blood-borne macrophages and dendritic cells (Fig. 2C, F, G and H). Therefore, in Orex-HA mice but not in littermate controls not expressing HA, the

Pandemrix® vaccine elicited a focal and protracted hypothalamic inflammation.

Interestingly, hypothalamus-infiltrating T cells were localized in the vicinity of orexinergic neurons in Orex-HA mice (Fig. 3A). In this inflammatory context, MHC class I molecules were detected at the plasma membrane and intracellularly in orexin<sup>+</sup> neurons (Fig. 3B). The tight apposition of T cells to orexinergic neurons (Fig. 3A), the expression of activated caspase 3 (Fig. 3C) and the detection of apoptotic nuclei (Fig. 3D and E) in some of the orexinergic neurons led us to investigate whether a loss of orexin-producing





**Figure 2** Pandemrix<sup>®</sup> vaccination following naïve CD4 and CD8 T-cell transfer elicits hypothalamic inflammation in Orex-HA mice. (A) Immunohistochemistry staining for CD3 (brown) in control and Orex-HA mice with a focus on the hypothalamus. Scale bar = 500  $\mu$ m and 100  $\mu$ m, respectively. (B) CD3+ T-cell density in the hypothalamus of control (blue triangles) and Orex-HA (red circles) mice 14 days after transfer of both HA-specific CD4 and CD8 T cells and vaccination with Pandemrix<sup>®</sup>. Results are expressed as mean  $\pm$  SEM of 10 to 14 mice per group from nine independent experiments. (C) Dimensionality reduction using the t-distributed stochastic neighbour embedding algorithm allows depicting immune subpopulations analysed by flow cytometry in the whole brain of control and Orex-HA mice, 2 weeks after transfer of HA-specific CD4 and CD8 T cells and Pandemrix<sup>®</sup> immunization. Represented cell populations result from manual gating based on the listed markers. (D) Quantification of the number of endogenous and transgenic T-cell infiltration within the whole brain of control and Orex-HA mice 14 days after Pandemrix<sup>®</sup> immunization. Results are expressed as mean  $\pm$  SEM of seven to eight mice per group from two independent experiments. (E) Numbers of infiltrating endogenous and transgenic T cells within dissected hypothalami of Orex-HA mice over time after Pandemrix<sup>®</sup> immunization. Results are expressed as mean  $\pm$  SEM of two to four pools of hypothalami for a total of seven to nine mice per time point. (F) Immunohistochemistry staining for Iba-1 (violet) and orexin-A (brown) in the hypothalamus of control and Orex-HA mice at Day 14. Scale bar = 50  $\mu$ m. (G) Representative histograms of MHC class II expression on myeloid cell subsets in the brain of control and Orex-HA mice 2 weeks after Pandemrix<sup>®</sup> immunization (H) Proportion of CD45<sup>dim</sup> CD11b<sup>+</sup> microglia expressing MHC class II molecules in control and Orex-HA mice, 14 days after Pandemrix<sup>®</sup> immunization. Box plots are depicting interquartile range and median, and whiskers extend between min and max values of 8 to 12 mice per group from three independent experiments. Statistical analyses were performed using the Mann-Whitney test. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

neurons would occur in vaccinated Orex-HA mice. We, therefore, quantified orexin-producing neurons in the lateral hypothalamus of Orex-HA recipient mice 2 months after Pandemrix<sup>®</sup> vaccination. Strikingly, an average 44% loss of orexinergic neurons was observed in the Orex-HA recipients as compared to control mice, whereas the neighbouring MCH-producing neurons were spared from destruction, suggesting an antigen-specific killing process (Fig. 3F). No significant differences in quantity of vigilance states and sleep architecture were found between vaccinated Orex-HA and control mice. Although no patent clinical manifestations were detected, polysomnographic analyses combined to video recordings revealed a cataplectic episode in two out of five vaccinated Orex-HA mice, but none in the four vaccinated control animals (Fig. 3G).

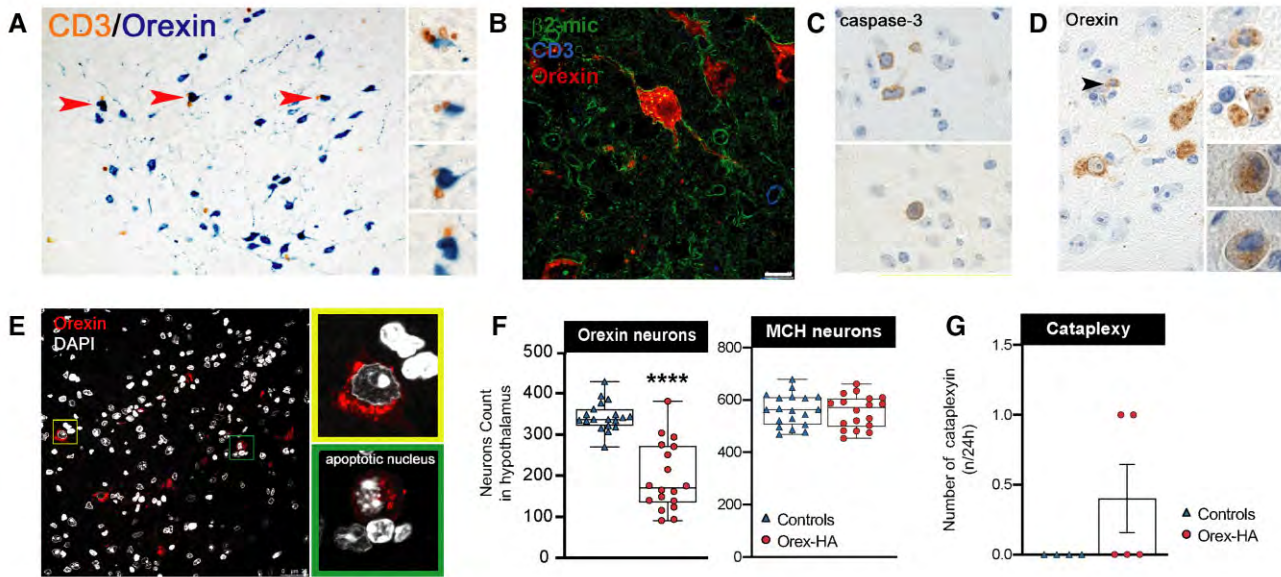
To evaluate whether the vaccine adjuvant, AS03, could also induce hypothalamic inflammation, Orex-HA mice and wild-type littermates received both HA-specific CD4 and CD8 T cells and were injected the next day with PBS, the AS03 adjuvant or the Pandemrix<sup>®</sup> vaccine. The full vaccine induced the phenotype described above, whereas no T-cell infiltration developed in the hypothalamus of PBS-injected Orex-HA recipient mice (Supplementary Fig. 3C). Immunization

with the AS03 adjuvant alone elicited inconstant and very modest hypothalamic T-cell infiltration in Orex-HA mice (Supplementary Fig. 3C), mostly located in the meninges, associated with limited microglial activation (Supplementary Fig. 3D and E). Furthermore, no degenerating Orexin+ neurons were detected in these mice ( $n = 3$  mice per group at Day 28).

Together, our data indicate that the HA-specific CD4 and CD8 T cells activated by the full Pandemrix<sup>®</sup> vaccine elicited hypothalamic inflammation only in mice expressing HA as a neo self-antigen. This antigen-dependent and focal inflammation resulted in the selective demise of orexinergic neurons, akin to human NT1.

### Molecular characterization of hypothalamus-infiltrating T cells in vaccinated Orex-HA mice

To identify the T cell subsets and molecular pathways potentially involved in the killing of orexinergic neurons in Pandemrix<sup>®</sup>-vaccinated Orex-HA mice, we cell-sorted activated (CD44<sup>high</sup> CD62L<sup>low</sup>) transferred (CD45.1+) and endogenous (CD45.1-) CD4 and CD8



**Figure 3** Hypothalamic inflammation induced by Pandemrix<sup>®</sup> vaccination of Orex-HA mice results in the selective loss of orexin-producing neurons. (A) Immunohistochemistry staining of CD3 (brown) and Orexin (blue) showing close contacts between T cells and orexinergic neurons (red arrow). Right insets are depiction of T cells in close apposition with orexinergic neurons in the hypothalamus of Orex-HA recipients. (B) Immunofluorescence staining for Orexin (red),  $\beta$ 2-microglobulin (green) and CD8 (blue) shows the presence of MHC-class I+ orexinergic neurons in the inflamed hypothalamus. (C) Images showing neurons with activated caspase-3, indicative of apoptosis. (D) Illustration of Orexin neurons showing orexin+ neurons with weak labelling for orexin (arrow head) and the presence of apoptotic nuclei as seen in the four photographs at higher magnification. (E) Immunofluorescence staining for Orexin (red) and nuclei (DAPI: white). Yellow inset (top) shows a normal orexinergic neuron while the green inset (bottom) shows a degenerating neuron with weak labelling for orexin and the presence of apoptotic bodies in the nucleus. (A–E) Photographs were taken in hypothalamus of Orex-HA animals 2 weeks after transfer of both HA-specific CD4 and CD8 T cells and Pandemrix<sup>®</sup> immunization. (F) Count of orexin- and MCH-producing neurons within the hypothalamus of control (blue triangles) and Orex-HA mice (red circles) 60 days after HA-specific CD4 and CD8 T-cell transfer and Pandemrix<sup>®</sup> immunization. Box plots are depicting interquartile range and median, and whiskers extend between min and max values of 18 to 19 mice per group from four independent experiments. Statistical analyses were performed using the Mann-Whitney test. \*\*\*\* $P < 0.0001$ . (G) Enumeration of cataplectic events in four control (blue triangles) and five Orex-HA mice (red circles) by polysomnographic analysis combined to video-monitoring.

T cells from dissected hypothalami, spleen and cervical lymph nodes at the peak of hypothalamic infiltration (Day 14 post-vaccination) (Supplementary Fig. 4A). RNA sequencing on these sorted T-cell populations allowed us to compare the gene expression profile of a given T-cell subset across different tissue sites and of different T-cell subsets in a given tissue.

The principal component analysis (PCA) showed that, while CD8 or CD4 T cells clustered separately as expected, the major variance was found when comparing hypothalamic T cells to their peripheral counterparts, and, intriguingly, hypothalamic endogenous and transferred CD8 or CD4 T cells exhibited closely related transcriptional signature (Fig. 4A). Indeed, a cluster dendrogram highlighted the transcriptomic proximity between hypothalamus-infiltrating CD8 T cells of both endogenous and transferred origin (Fig. 4B). We identified 1247 DEG when comparing transferred CD8 T cells from the hypothalamus to those from the spleen. A similar analysis yielded 1333 DEG for the endogenous CD8 T cells (Supporting Data S1). Over representation analysis of the DEG highlighted KEGG pathways involved in immune responses and inflammatory diseases, most notably cytokine/cytokine receptor interaction (Fig. 4C). As illustrated in the heat map (Fig. 4D), transferred HA-specific CD8 T cells in the hypothalamus were characterized by an activated phenotype (*Il2ra*, *Ctla4*, *Pdcd1*, *Tigit*, *CD38*, *Irf8*, *Fos*, *Jun*, etc), with tissue-migration properties (*Sell*, *Jam2*, *Mmp2*) and an inflammatory and cytotoxic potential (*Ifng*, *CCL3*, *CCL4*, *Csf2*, *Gzmb*, *FasL*).

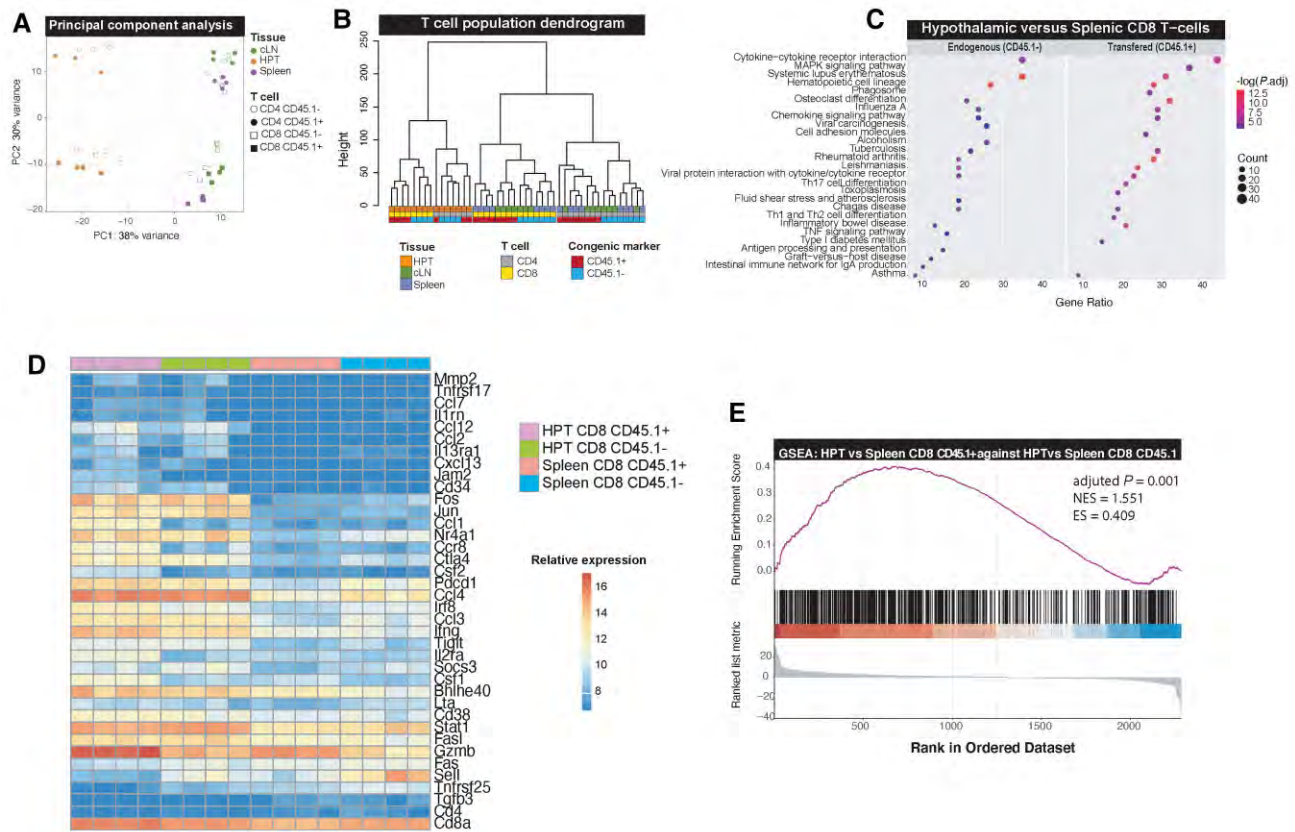
The hypothalamus-infiltrating endogenous CD8 T cells and the transferred CD8 T cells shared 345 DEG when compared to their splenic counterparts. Gene-set enrichment analyses

revealed that the transcriptomic signature of transferred CD8 T cells in the hypothalamus, relative to their peripheral counterparts, was largely shared by hypothalamic endogenous CD8 T cells (Fig. 4E). This similarity was also apparent when analysing the expression of selected genes (Fig. 4D). These data suggest that both endogenous and transferred CD8 T cells harbor a pathogenic potential.

The quantitatively modest transferred CD4 T cells found within the hypothalamus was also characterized by DEG involved in the highlighted KEGG pathways related to immune responses and inflammatory or infectious diseases (Supplementary Fig. 4B). Interestingly, this hypothalamic CD4 T cell subset expressed increased levels of cytotoxic and inflammatory transcripts (*Tnf*, *Tnfsf10*, *FasL*, *Gzmb*, *Ifng*, *Csf2*, *CXCL10*, *CCL3*, *CCL4*), but also transcripts related to immune regulation and tissue repair (*IL-10*, *IL-10ra*, *Areg*) as compared to CD4 T cells residing in secondary lymphoid organs (Supplementary Fig. 4C).

### Respective role of HA-specific CD4 and CD8 T cells in the pathogenic process

Since the RNAseq analyses showed that hypothalamus-infiltrating CD8 and CD4 T cells expressed genes involved in effector functions, we assessed which T-cell subsets were necessary to elicit hypothalamic inflammation. Orex-HA mice were transferred with either HA-specific CD4 or CD8 T cells, or not transferred prior to Pandemrix<sup>®</sup> vaccination. The endogenous T-cell repertoire of Orex-HA mice was insufficient *per se* to induce focal hypothalamic



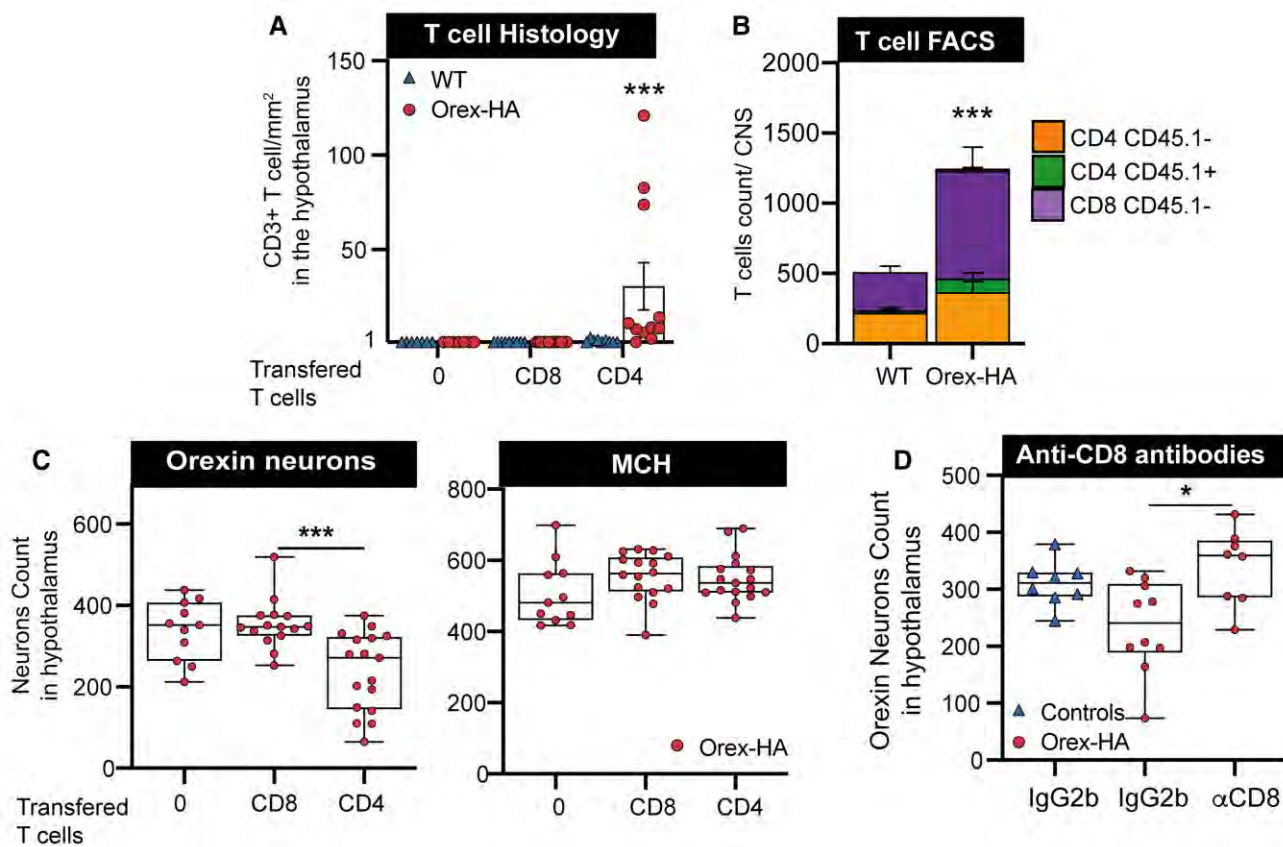
**Figure 4** Analysis of the gene expression profile of hypothalamus-infiltrating T cells compared to that of their peripheral counterparts. (A) PCA of the samples originating from hypothalamus (Hypo), cervical lymph nodes (cLN) and spleen. PC1 displays 38% variance that can be explained by the separation of the three tissues. PC2 shows 30% variance that can be explained by the separation between the CD4 and CD8 T-cell subsets. (B) Dendrogram of all samples displaying the gene expression proximity of the samples according to T-cell subtype and location. Height has been calculated as the mean Euclidean distance between two sets of leaves. (C) Top 20 over-representation analysis KEGG pathways generated from the DEGs (Padj. cut-off <math><0.05</math> and fold change >1.5) between hypothalamus versus spleen endogenous CD8 T cells (left) or transferred CD8 T cells (right). (D) Heat map of genes of interest chosen from the top KEGG pathways. (E) GSEA plot showing the enrichment of the DEGs from hypothalamus versus spleen transferred CD8 T cells within the DEGs from hypothalamus versus spleen endogenous CD8 T cells. ES = enrichment score; NES = normalized enrichment score.

inflammation after vaccination (Fig. 5A). In Orex-HA mice supplemented only with HA-specific CD8 T cells, the transferred CD8 T cells initially underwent an intense proliferation and differentiation, similar to that observed in the presence of transferred CD4 T cells (Supplementary Fig. 5A–D). However, the subsequent functions of these ‘unhelped’ HA-specific CD8 T cells were altered as they failed to induce hypothalamic T-cell infiltration in Orex-HA mice after Pandemrix® vaccination (Fig. 5A). Consequently, the density of orexin+ neurons remained in the normal range in Orex-HA mice, not transferred with T cells or transferred only with CD8 T cells prior to Pandemrix® vaccination (Fig. 5C). This contrasted with the data shown above in which co-transfer of HA-specific CD8 T cells with HA-specific CD4 T cells resulted in consistent hypothalamic T-cell infiltration (Fig. 2B), including 32% of transferred HA-specific CD8 T cells (Fig. 2E), leading to significant loss of orexin+ neurons (Fig. 3F).

Interestingly, Orex-HA mice supplemented only with HA-specific CD4 T cells developed hypothalamic T-cell infiltration of varying magnitude that, however, remained significantly less than what was detected in mice supplemented with both HA-specific CD4 and CD8 T cells (Figs 5A and 2B). Endogenous CD8 T cells dominated this infiltration (Fig. 5B). Importantly, the density of orexin+ neurons in Orex-HA mice receiving HA-specific CD4 T cells

was decreased by 33% on average, whereas MCH+ neurons were spared (Fig. 5C).

These data indicate that transferred HA-specific CD4 T cells are necessary for T-cell infiltration in the hypothalamus of vaccinated Orex-HA mice and for the subsequent selective destruction of orexinergic neurons. The transferred HA-specific CD4 T cells could be either directly pathogenic or act indirectly through another immune cell subset. To address this question, given that the endogenous hypothalamic CD8 T cells exhibited a transcriptomic profile similar to that of ‘pathogenic’ transgenic HA-specific CD8 T cells, we further assessed whether depletion of CD8 T cells would prevent the destruction of orexinergic neurons induced by the Pandemrix® vaccination in Orex-HA mice supplemented with HA-specific CD4 T cells. Antibody-mediated depletion of CD8 T cells was started 5 days after Pandemrix® vaccination, prior to parenchymal T-cell infiltration of the hypothalamus. This treatment resulted in a clear reduction in the number of CD8 T cells infiltrating the hypothalamus, with no reduction of endogenous and transferred CD4 T cells (Supplementary Fig. 5E and F). Importantly, the reduction of orexinergic neuron density induced by transferred HA-specific CD4 T cells and vaccination was significantly prevented by antibody-mediated CD8 T-cell depletion (Fig. 5D). These data indicate that CD8 T cells are necessary in mediating neuronal damage and that



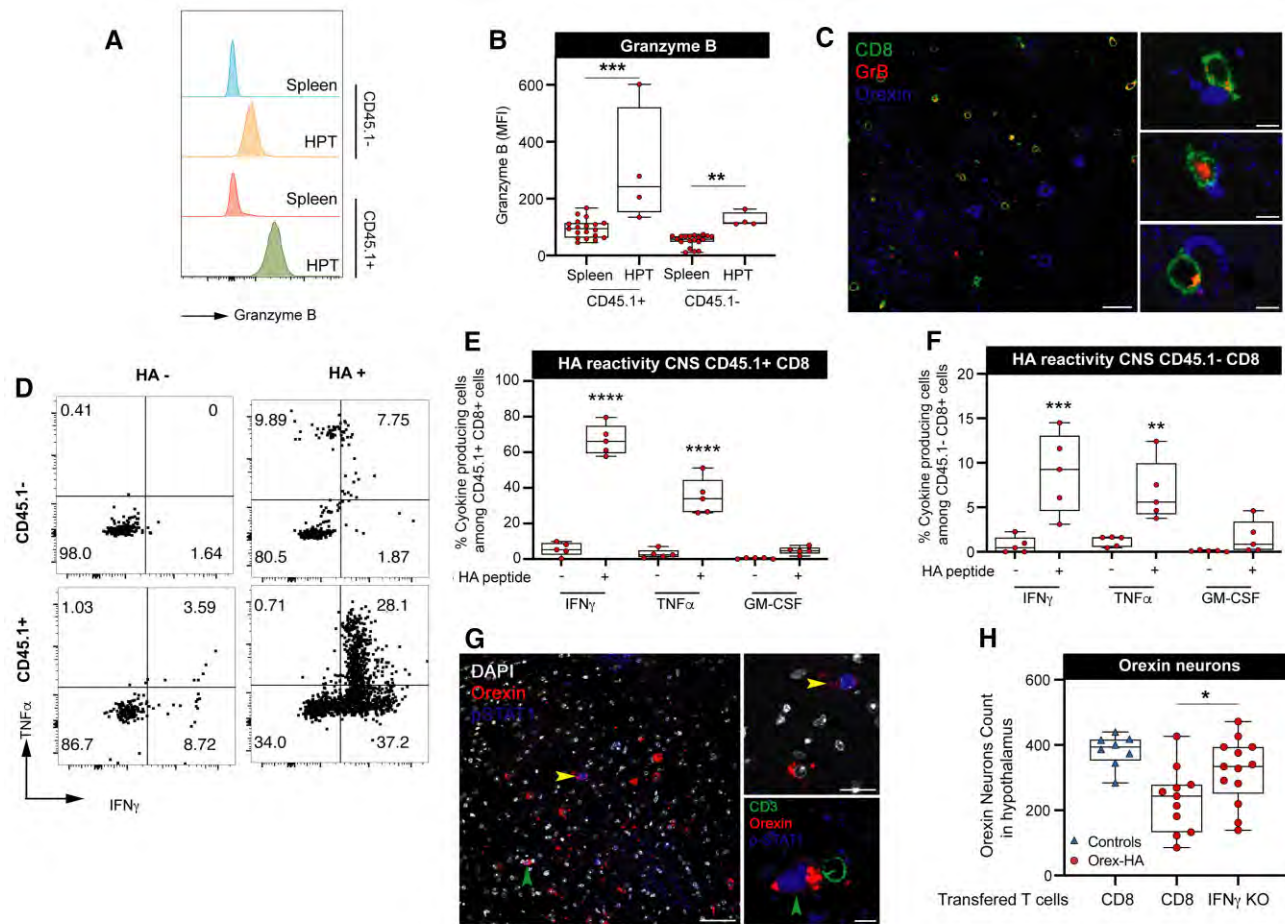
**Figure 5** The loss of orexinergic neurons induced by transferred CD4 T cells upon Pandemrix<sup>®</sup> immunization requires either transferred or endogenous CD8 T cells. (A) CD3 T cell density in the hypothalamus of control (blue triangles) and Orex-HA (red circles) mice transferred with no T cells (0), HA-specific CD8 T cells, or HA-specific CD4 T cells, 14 days after Pandemrix<sup>®</sup> immunization. Results are expressed as mean  $\pm$  SEM of 7 to 13 mice per group from four independent experiments. (B) Numbers of infiltrating endogenous and transgenic T cells within the whole CNS of control or Orex-HA mice transferred with HA-specific CD4 T cells only, 14 days after Pandemrix<sup>®</sup> immunization. Results are expressed as mean  $\pm$  SEM of 8 to 10 mice per group from two independent experiments. (C) Count of orexin- and MCH-producing neurons within the hypothalamus of Orex-HA mice (red circles) 60 days after transfer of either no T cells (0), HA-specific CD8 T cells or HA-specific CD4 T cells and Pandemrix<sup>®</sup> immunization. Box plots are depicting interquartile range and median, while whiskers extend between min and max values of 11 to 17 mice per group. (D) Orexin neuron count within the hypothalamus of control (blue triangle) or Orex-HA mice (red circles) 4 weeks after transfer of HA-specific CD4 T cells, Pandemrix<sup>®</sup> immunization and treatment with either a depleting anti-CD8 antibody or control IgG2b from Day 5 onwards. Results are from two independent experiments. Statistical analyses were performed using the Mann–Whitney test. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

the endogenous CD8 T-cell population can substitute for the transgenic HA-specific CD8 T cells. Collectively, the data suggest a step-wise scenario in which transferred HA-specific CD4 T cells are necessary for the entry of CD8 T cells in the hypothalamus of vaccinated Orex-HA mice, whereas CD8 T cells act as the final actors of orexinergic neuronal loss.

### Essential role for IFN $\gamma$ produced by CD8 T cells in the pathogenic process

We characterized further the endogenous T-cell populations infiltrating the hypothalamus of vaccinated Orex-HA mice supplemented with both HA-specific CD4 and CD8 T cells. Based on TCR gene segment usage and rearrangement extracted from the RNAseq datasets, the endogenous CD4 and CD8 T-cell compartments appear diverse (Supplementary Fig. 6A–D). The diversity of the endogenous CD4 T-cell compartment was further illustrated by the presence of sizeable proportion of Foxp3-expressing regulatory cells (Supplementary Fig. 6E), as well as a significant subset responding to HA peptide stimulation by production of pro-inflammatory cytokines such as IFN $\gamma$ , TNF $\alpha$  and GM-CSF (Supplementary Fig. 6F).

We then investigated the activation status and the mechanisms whereby CD8 T cells destroy the orexin+ neurons. In line with the transcriptomic data (Fig. 4D), both transferred and endogenous CD8 T cells produced higher levels of granzyme B in the hypothalamus than in the spleen (Fig. 6A and B). Of note, *in situ* granzyme B polarization from CD8 T cells towards orexin-producing neurons was detected in vaccinated Orex-HA mice (Fig. 6C), suggesting a direct cytotoxic effect of the tissue-infiltrating CD8 T cells following TCR:MHC-peptide interaction. Moreover, a majority of transferred and endogenous CD8 T cells, and a large proportion of CD4 T cells, from the hypothalamus produced high levels of inflammatory cytokines upon polyclonal activation (Supplementary Fig. 7A and B). A sizable fraction of endogenous (on average 29%) and transferred (on average 11.4%) CD8 T cells expressed the cell proliferation-associated Ki67 marker, suggesting *in situ* antigen stimulation. This led us to evaluate, by fluorescence activated cell sorting, whether hypothalamus-infiltrating CD8 T cells responded to HA antigen (Fig. 6D). As expected, a strong antigen-specific response was elicited in transferred (CD45.1<sup>+</sup>) CD8 T cells (Fig. 6E). Notably, a distinct subset of endogenous (CD45.1<sup>-</sup>) CD8 T cells was able to produce IFN $\gamma$  and TNF $\alpha$  upon *ex vivo* stimulation with an



**Figure 6** CD8 T cells act as final effectors of orexinergic neuron destruction through an IFN $\gamma$ -dependent pathway. (A) Representative histogram and (B) quantification of granzyme B expression in transferred (CD45.1+) and endogenous (CD45.1-) CD8 T cells from the hypothalamus and spleen of Orex-HA mice 14 days after transfer of both HA-specific CD4 and CD8 T cell and Pandemrix<sup>®</sup> immunization. Box plots are depicting interquartile range and median, while whiskers extend between min and max values. Individual data points are plotted for spleen ( $n = 19$  mice) while pools of four to eight mice were used for hypothalamus. (C) Confocal fluorescence staining of CD8 (green), Granzyme B (red) and Orexin (blue) shows the distribution of cytotoxic T cells in the lateral hypothalamus of Orex-HA mice 14 days after HA-specific T-cell transfer and Pandemrix<sup>®</sup> immunization. Scale bar = 25  $\mu\text{m}$ . The three enlargements on the right show GrB+ CD8 T cells attached to Orexin+ neurons or processes. Scale bars = 5  $\mu\text{m}$ . (D–F) Representative fluorescence activated cell sorting plot (D) and quantification of production of IFN $\gamma$ , TNF $\alpha$  and GM-CSF upon HA stimulation of transferred CD45.1+ (E) and endogenous CD45.1- (F) CD8 T cells from the hypothalamus of Orex-HA mice 28 days after transfer of HA-specific T cell and Pandemrix<sup>®</sup> immunization. Box plots depict interquartile range and median, while whiskers extend between minimum and maximum values of five mice per group from two independent experiments. (G) Overview of the orexinergic nucleus stained for Orexin (red), p-STAT-1 (blue) and nuclear counterstain DAPI (white). Neurons indicated by the yellow and green arrowhead show upregulation of p-STAT-1. Scale bar = 50  $\mu\text{m}$ . The top inset shows an enlargement of the neuron indicated by yellow arrowhead. Scale bar = 20  $\mu\text{m}$ . The bottom inset shows the p-STAT-1+ orexin+ neuron, indicated by the green arrowhead, combined with a staining for CD3+ T cell. Scale bar = 10  $\mu\text{m}$ . (H) Orexin neurons count 60 days after transfer of HA-specific CD4 T cells in combination with either IFN $\gamma$ -deficient or IFN $\gamma$ -proficient HA-specific CD8 T cells. Box plots depict interquartile range and median, whiskers extend between minimum and maximum values of 8 to 13 mice per group from two independent experiments. Statistical analyses were performed using the Mann-Whitney U-test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

immunodominant HA peptide (Fig. 6F). The identification of orexinergic neurons expressing a phosphorylated form of STAT1 (an indirect marker of IFN $\gamma$  receptor activation) in vaccinated Orex-HA mice suggested IFN $\gamma$  signalling within neurons (Fig. 6G). Since neurons can express MHC class I molecules upon IFN $\gamma$  stimulation, we wondered whether the strong IFN $\gamma$  production by hypothalamus-infiltrating CD8 T cells could fuel the pathogenic process. We, therefore, transferred Orex-HA mice with HA-specific CD4 T cells and either wild-type or IFN $\gamma$ -deficient HA-specific CD8 T cells prior to Pandemrix<sup>®</sup> vaccination. Although hypothalamic T-cell infiltration was similar in both groups at day 30 (IFN $\gamma$ -proficient CD8 T cells,  $n = 4$ ,  $m = 17.67 \pm 5.15/\text{mm}^2$  vs. IFN $\gamma$ -KO CD8 T cells,  $n = 6$ ,  $m = 17.75 \pm 5.75/\text{mm}^2$ ;

$P = 0.99$ ), a significant reduction in the loss of orexin-producing neurons was observed at day 60 (Fig. 6H). These data indicate that CD8 T cell-derived IFN $\gamma$  plays an important role in neuronal loss in the animal model.

## Discussion

In this work, we illuminate the immune mechanisms potentially linking Pandemrix<sup>®</sup> vaccination to hypothalamic inflammation and the eventual loss of orexin-producing neurons, a pathognomonic feature of NT1. Given the overwhelming genetic evidence indicating a role of T cells in human narcolepsy, we focused on this cell type using a dedicated animal model. Pandemrix<sup>®</sup> vaccination

of Orex-HA mice enriched in naïve HA-specific CD4 and CD8 T cells resulted in hypothalamic inflammation and selective destruction of orexin<sup>+</sup> neurons. Transcriptomic analysis of both transferred and endogenous CD4 and CD8 T cells from hypothalamus or secondary lymphoid organs highlighted the remarkable singularity of hypothalamus-infiltrating endogenous and transferred T cells. The contribution of each T cell subset in the development of this pathogenic process was investigated using transfer of single populations of T cells as well as antibody-mediated depletion of given T-cell populations. Transferred HA-specific CD4 T cells were necessary for the development of hypothalamic inflammation but required CD8 T cells, either endogenous or adoptively transferred, to carry out the destruction of orexinergic neurons. The propensity of hypothalamic CD8 T cells to produce IFN $\gamma$  in response to HA peptide stimulation and the indication that IFN $\gamma$  signalling was operating in orexinergic neurons pointed to a role for IFN $\gamma$  released by T cells in pathogenesis. This work demonstrates that an immunopathological process mimicking narcolepsy can be elicited by the Pandemrix<sup>®</sup> vaccine in an experimental mouse model. Our work also highlights the synergy between autoreactive CD4 and CD8 T cells for disease development: CD4 T cells being essential for the development of hypothalamic inflammation and CD8 T cells being responsible for the final execution of orexin-producing neurons.

Molecular mimicry is usually suspected when an autoimmune process develops following a documented infection or vaccination.<sup>26</sup> This mechanism has been confirmed for a number of neurological autoimmune diseases, such as Guillain-Barré syndrome occurring after *Campylobacter jejuni* infection, in which antibodies against bacteria lipo-oligosaccharides cross-react with peripheral nerve gangliosides<sup>27</sup> or Sydenham's chorea related to a cross-reactivity between Group A streptococcal epitopes and neuronal antigens.<sup>28</sup> Related to this mechanism, paraneoplastic diseases can develop as a result of *de novo* expression of a (neuronal) autoantigen by tumoral cells leading to activation of pathogenic autoreactive T cells.<sup>29</sup> More broadly, instances in which self-reactive T-cell clones from individuals with autoimmune diseases are shown to cross-react with multiple foreign or even self-antigens are now becoming uncountable.<sup>30,31</sup> Molecularly, this observation is related to the overall promiscuity of MHC-peptide recognition by a given TCR. However, to our knowledge, there are no proven instances of pathogenic cross-reactivity between a vaccine viral antigen and a human self-antigen. The search for molecular mimicry in post-Pandemrix<sup>®</sup> narcolepsy has been active. A striking study reported detection of autoantibodies against the orexin receptor type 2 in 85% of post-Pandemrix<sup>®</sup> NT1 paediatric patients in Finland ( $n=20$ ), which cross-reacted with the flu vaccine nucleoprotein.<sup>32</sup> Similar data were not replicated in other patient cohorts.<sup>33</sup> Interestingly, although cross-reactivity was not functionally demonstrated, TCR sequences of CD4 T cell clones recognizing C-amidated forms of orexin peptides in association with HLA-DQ6 shared striking similarities with those from influenza HLA-DQ6:HA-specific clones.<sup>19</sup> These data speak in favour of a molecular mimicry mechanism underlying the development of narcolepsy in persons vaccinated with Pandemrix<sup>®</sup> during the 2009/2010 pandemic. Yet they do not prove causality. Our data go one step further by showing that orexinergic neuron destruction may result from molecular mimicry between the vaccine HA antigen and a similar/identical antigen expressed primarily in orexinergic neurons. Nevertheless, we did not test in our mouse model whether exposure to the infectious H1N1 'influenza' virus could elicit, or contribute to, a similar phenotype.<sup>34,35</sup>

Our previous study showed that adoptively transferred autoreactive Th1 cells could not promote orexin<sup>+</sup> neuron death despite their presence in the hypothalamus, whereas transfer of cytotoxic CD8 T cells could induce both inflammation and death of orexin<sup>+</sup> neurons.<sup>16</sup> The current work suggests a subtler role for autoreactive CD4 T cells, elicited by the flu vaccine. Indeed, CD4 T cells were essential to endow the autoreactive CD8 T cells with the capacity to enter the hypothalamus. In their absence, transferred CD8 T cells proliferated strongly but were harmless, presumably because the endogenous CD4 T cell help induced by the vaccine was limiting. Although these 'unhelped' transferred CD8 T cells acquired an antigen-experienced phenotype and could produce IFN $\gamma$ , their functional properties were likely altered as they failed to penetrate and/or remain in the hypothalamic parenchyma. These data are in line with previous data underlining the importance of CD4 T cell help in conferring tissue invasiveness of CD8 T cells recognizing the same immunizing antigen, through up-regulation of chemokine receptors and matrix metalloproteases.<sup>36</sup> In addition, following vaccination in absence of CD4 T cell help, vaccine-specific CD8 T cells exhibited decreased cytotoxic potential and expressed higher levels of co-inhibitory receptors such as PD-1, LAG-3, Tim-3, 2B4 and BTLA.<sup>36–38</sup> CD4 T cell help is mostly provided through activation of dendritic cells that relay CD28 and CD27-mediated signals in CD8 T cells.<sup>39</sup> In our mouse model, both transferred and endogenous CD8 T cells expressed high levels of granzyme B, FasL, IFN $\gamma$  and CCL3, CCL4 in the hypothalamus. This is indicative of a pro-inflammatory and cytotoxic potential. Moreover, evidence for granzyme-mediated cytotoxicity of the targeted neurons was provided by neuropathological analyses. Then, once in the hypothalamus, converging evidence indicate that the cytotoxic CD8 T cells mediate antigen-specific killing of orexinergic neurons. The concept that antigen-specific T cells can directly target CNS neurons has received strong support from experimental models of CNS infection or autoimmunity.<sup>40–43</sup> Moreover, accumulating data strongly suggest that a similar mechanism could be at play in human inflammatory or degenerative neurological diseases, including NT1 but also amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease.<sup>42,44–49</sup> One unexpected result was the observation that the transferred CD4 T cells could also promote neuronal cell death, in the absence of transferred HA-specific CD8 T cells, by licensing the endogenous CD8 T cells to kill. Indeed, the endogenous CD8 T-cell compartment contains HA-specific T cells likely elicited by the Pandemrix<sup>®</sup> vaccine; these cells, as shown by the CD8 T cell-depletion experiments, were necessary for orexinergic neuron loss. The contribution of the endogenous CD4 T-cell population infiltrating the hypothalamus to the disease process is less clear. Indeed, this subset is composed of at least three different populations: (i) HA-specific CD4 T cells that could very well contribute to the inflammation and destruction of orexinergic neurons; (ii) bystander activated CD4 T cells of unrelated specificity that have the propensity to release IFN $\gamma$  and TNF within the hypothalamus and could, thereby, contribute to the pathogenic process as suggested in the context of experimental autoimmune encephalomyelitis<sup>50</sup>; and (iii) regulatory CD4 T cells expressing Foxp3 that are recruited locally.

Our results are in line with the strong association of NT1 with the HLA-DQA1\*01:02-HLA-DQB1\*06:02 haplotype and with the increased frequency of orexin-specific CD4 T cells found in the blood of NT1 patients versus healthy donors.<sup>18,19</sup> Indeed, the increased number of autoreactive CD4 T cells can contribute to break the mechanisms of peripheral immune tolerance thereby enabling CD8 T cells to migrate within the hypothalamus.

T cells can release IFN $\gamma$  directionally, promoting molecular interactions with cells presenting the recognized MHC-peptide ligands.<sup>51</sup> This event could well occur in the hypothalamus of our mouse model, as orexinergic neurons exhibit nuclear localization of phosphorylated STAT1, an indirect indication of IFN $\gamma$ -R triggering. Other investigators have observed expression of pSTAT1 in neurons in experimental models of CD8 T cell-mediated experimental CNS autoimmune diseases as well as in human diseases associated with prominent CD8 T cell brain inflammation.<sup>40,43</sup> As a result of IFN $\gamma$  signalling, CNS neurons can engage several pro-inflammatory pathways that will culminate in disruption of neuronal connectivity and neuronal death. First, neurons can release chemokines, such as CCL2 and CXCL10, which will attract myeloid cells as well as additional T cells in their vicinity. Second, IFN $\gamma$  signalling promotes MHC class I expression by neurons, hence promoting their recognition by antigen-specific cytotoxic CD8 T cells. Finally, autocrine or paracrine action of IFN $\gamma$  enhances *in vivo* mobility of cytotoxic lymphocytes and promotes target killing.<sup>52</sup> The importance of this pathway is underlined in our model by the preventive impact of IFN $\gamma$  deficiency on the loss of neurons but not on CD8 T cell penetration in the hypothalamus. The importance of the IFN $\gamma$  pathway in pathogenesis is reinforced by data from patients with NT1. Indeed, serum levels of IFN $\gamma$  were higher in NT1 patients, in particular those with recent onset, as compared to healthy controls,<sup>53</sup> although this was not detected in CSF.<sup>54</sup> Intriguingly, the serum levels of CXCL9 and CXCL10, two IFN $\gamma$ -induced chemokines were higher in post-H1N1 vaccine NT1 as compared to non-exposed NT1 patients.<sup>53</sup> Moreover, higher frequencies of orexin-specific T cells able to produce IFN $\gamma$  were detected in patients with NT1 as compared to healthy controls.<sup>18,55</sup> Based on these observations, we surmise that IFN $\gamma$  neutralization could represent an attractive therapeutic strategy for neurological autoimmune diseases including, but not restricted to, NT1. However, this therapeutic approach would have to be tested at the onset of disease before major and irreversible destruction of neuronal populations occur.

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## Competing interests

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## Supplementary material

Supplementary material is available at *Brain* online.

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Supplementary Data for:

## **Influenza vaccination induces autoimmunity against orexin neurons in a mouse model for narcolepsy**

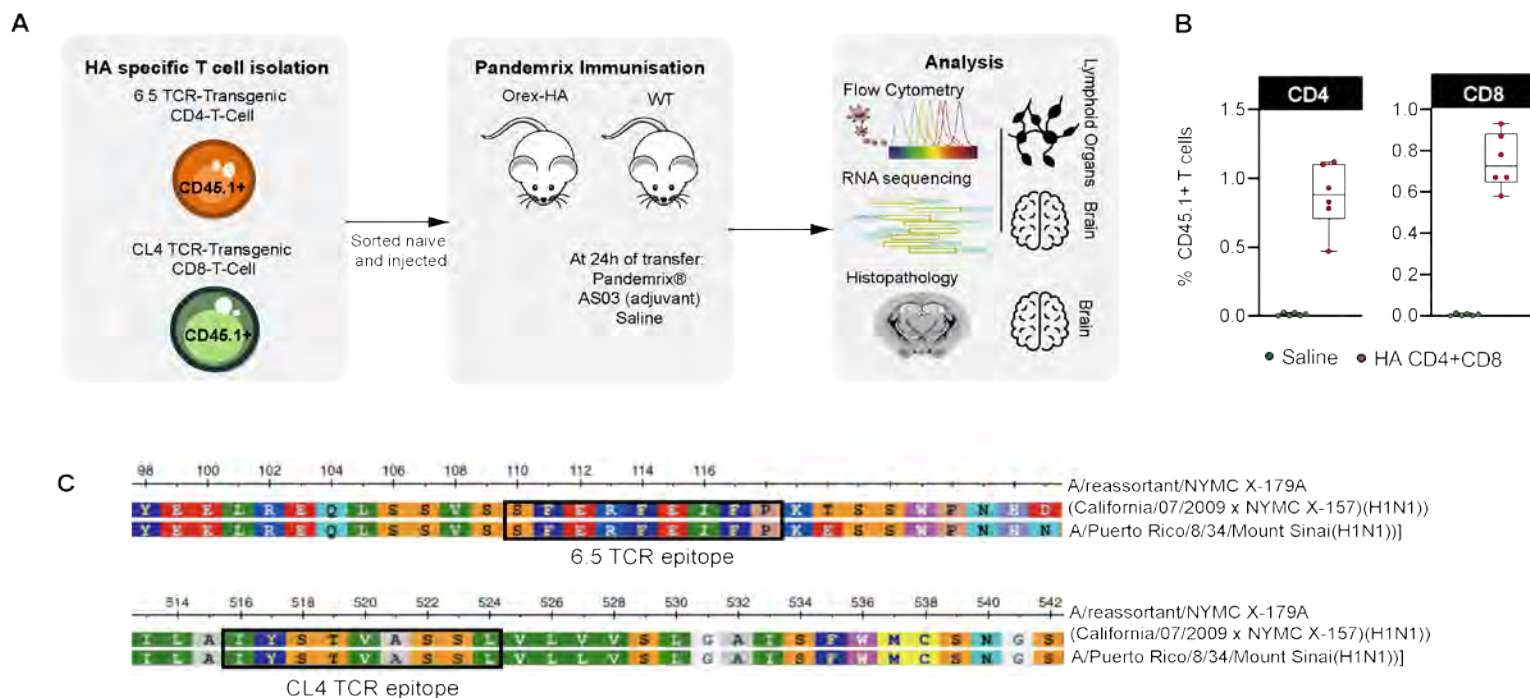
Raphaël Bernard-Valnet <sup>1,2, †</sup>, David Frieser <sup>1, †</sup>, Xuan-Hung Nguyen <sup>1,3, †</sup>, Leila Khajavi <sup>1</sup>, Clémence Quériault <sup>1</sup>, Sébastien Arthaud <sup>4</sup>, Silvia Melzi <sup>4</sup>, Maxime Fusade-Boyer <sup>5</sup>, Frederick Masson <sup>1</sup>, Matthias Zytnicki <sup>6</sup>, Abdelhadi Saoudi <sup>1</sup>, Yves Dauvilliers <sup>7</sup>, Christelle Peyron <sup>4</sup>, Jan Bauer <sup>7,8</sup>, and Roland S. Liblau <sup>1,8</sup>

<sup>†</sup>These authors contributed equally to this work.

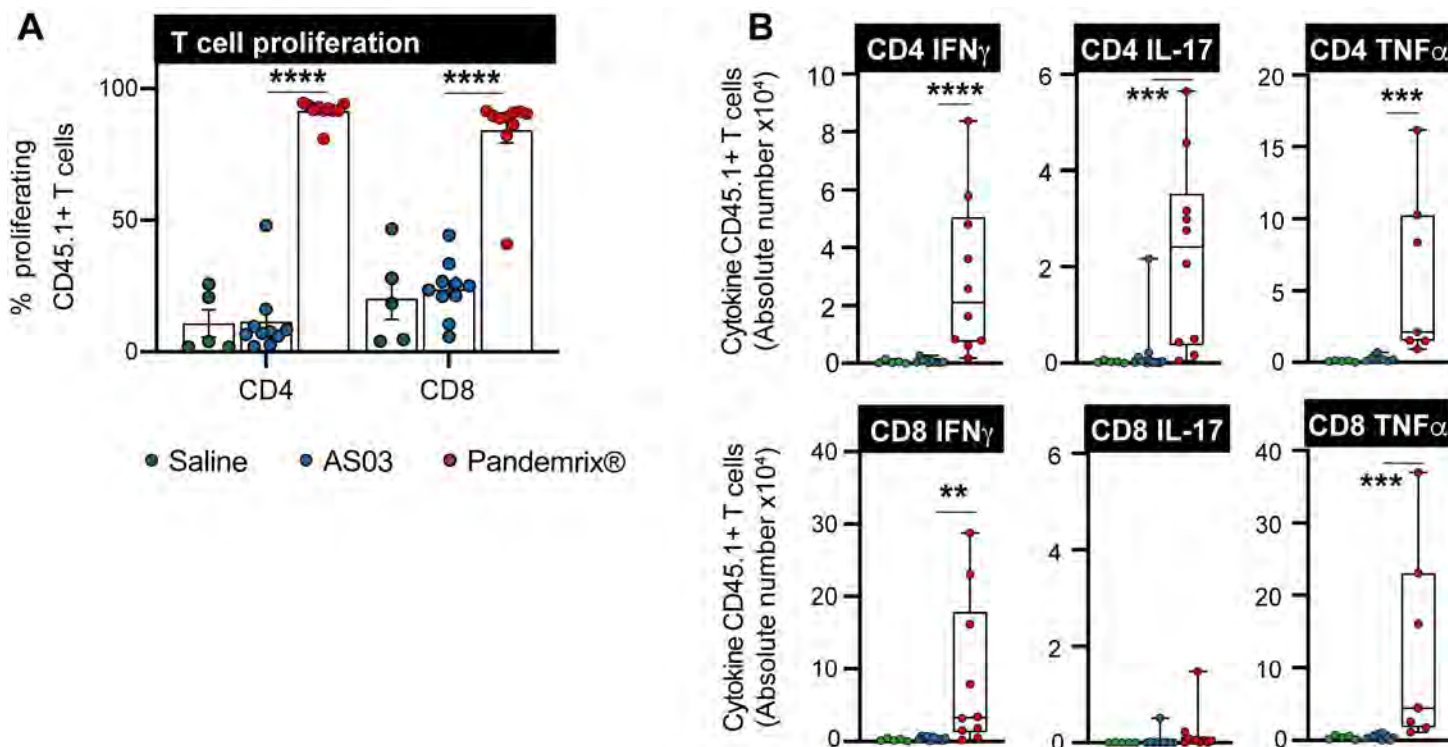
### **Author affiliations:**

1. Toulouse Institute for infectious and inflammatory diseases (Infinity), University of Toulouse, CNRS, INSERM, UPS, Toulouse, France
2. Service of neurology, clinical neurosciences department, Lausanne university hospital and Lausanne university, Lausanne, Switzerland
3. Vinmec Institute of Applied Science and Regenerative Medicine, Vinmec Healthcare System, Hanoi, Vietnam
4. INSERM U1028, CNRS UMR 5292, Center for Research in Neuroscience, University of Lyon 1, Bron, France
5. IHAP, UMR1225, Université de Toulouse, INRAe, ENVT, Toulouse, France
6. Unité de Mathématiques et Informatique Appliquées, INRAE, Castanet-Tolosan, France
7. National Reference Center for Orphan Diseases, Narcolepsy, Idiopathic hypersomnia and Kleine-Levin Syndrome, Department of Neurology, Gui-de-Chauliac Hospital, CHU de Montpellier, INSERM U1061, Montpellier, France
8. Center for Brain Research, Medical University of Vienna, Vienna, Austria
9. Department of Immunology, Toulouse University Hospitals, Toulouse, France

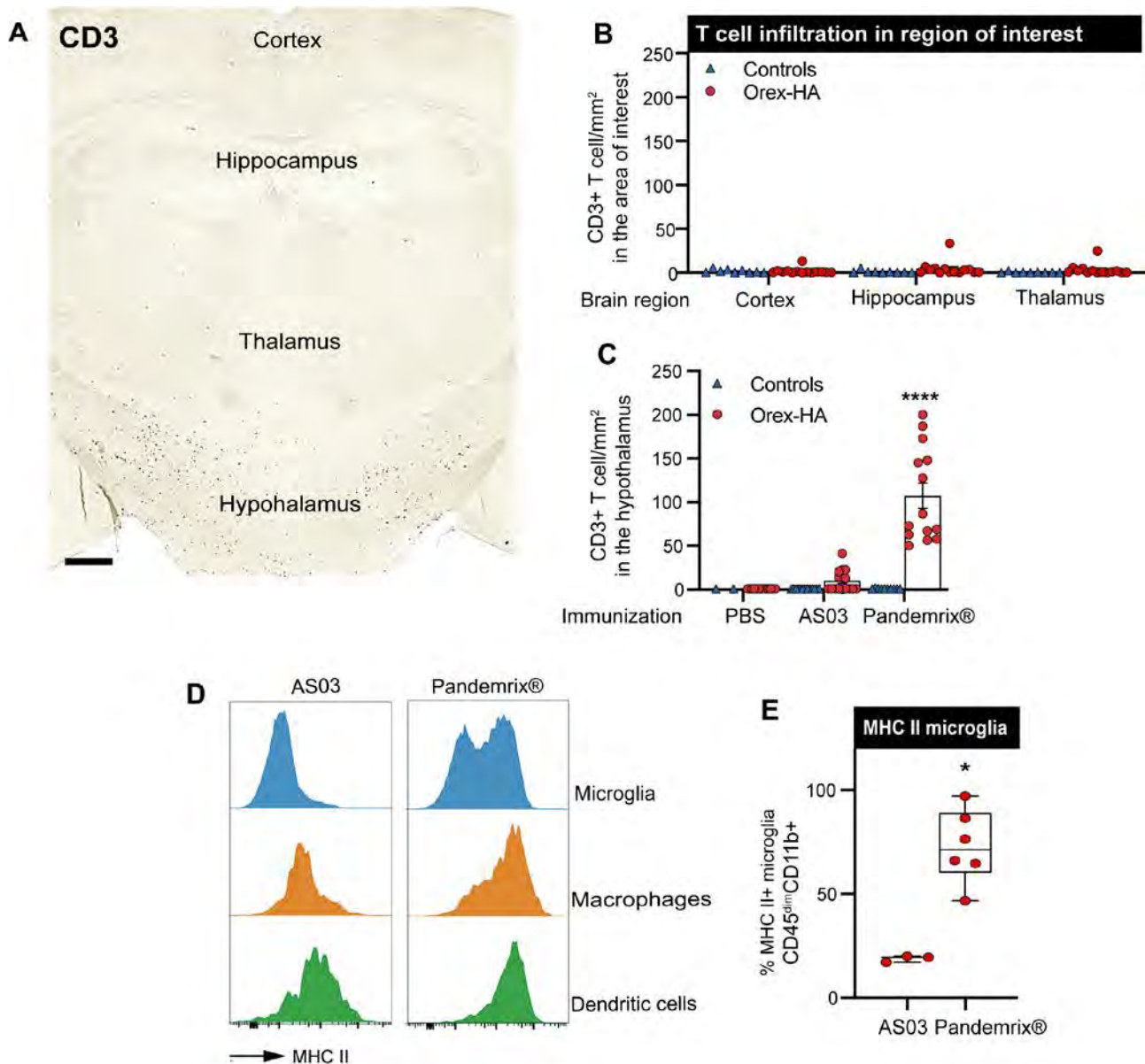
Correspondence to: Prof. Roland Liblau;  
Toulouse Institute for infectious and inflammatory diseases,  
CHU Purpan BP 3028, 31024 Toulouse cedex 03, Toulouse, France  
Email: roland.liblau@inserm.fr



**Fig. S1. Experimental design and Hemagglutinin sequence homology at sites recognized by the TCR-transgenic T cells.** (A) Schematic depiction of the experimental design and analyses performed in this study. (B) The proportion of transferred (CD45.1<sup>+</sup>) HA-specific T cells in the splenic CD4 and CD8 T cell compartments was assessed by flow cytometry prior to Pandemrix<sup>®</sup> vaccination. (C) HA amino-acid sequences are fully shared for epitopes recognized by the K<sup>d</sup>-restricted CL4-TCR and the I-E<sup>d</sup>-restricted 6.5-TCR transgenic T cells between the H1N1 influenza A virus (A/Puerto Rico/8/34/Mount Sinai) strain and the strain contained in Pandemrix<sup>®</sup> (Influenza A virus (A/reassortant/NYMC X-179A (California/07/2009 x NYMC X-157))).

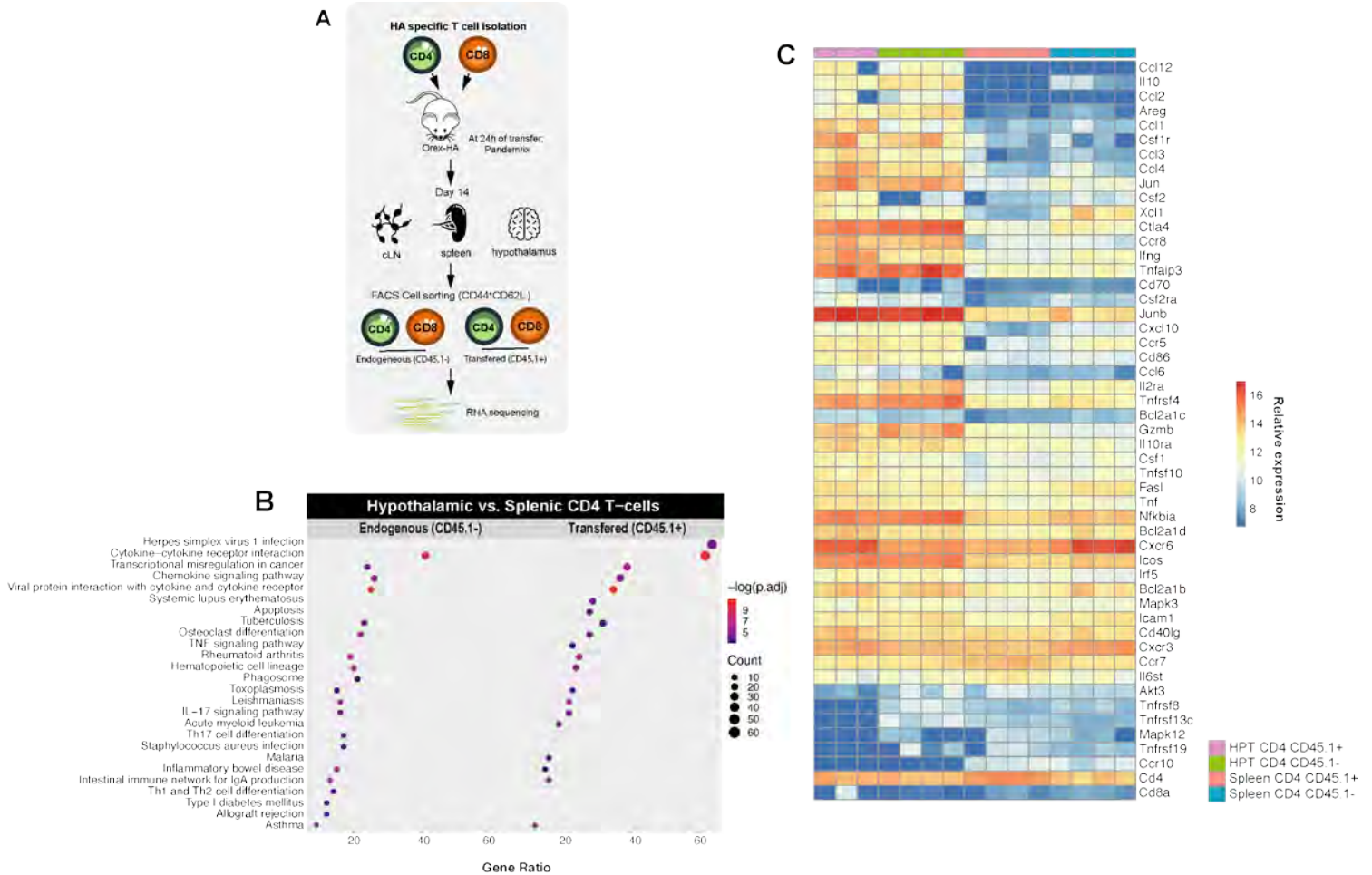


**Fig. S2. Activation of HA-specific T cells in draining lymph nodes of control animals upon Pandemrix® immunization.** (A) Following transfer in control recipient mice (not expressing HA), naïve CD45.1+ HA-specific CD4 and CD8 T cells have extensively proliferated 5 days after immunization with Pandemrix® (red circles) but not after injection of saline (green circles) or AS03 adjuvant (blue circles). Results are expressed as mean  $\pm$  SEM of 5 to 10 mice per group from 5 independent experiments. (B) Absolute numbers of CD45.1+ HA-specific CD4 and CD8 T cells producing IFN $\gamma$ , IL-17, or TNF $\alpha$ , 5 days after injection of either saline, AS03, or Pandemrix®. Boxplots are depicting interquartile range and median, while whiskers extend between min and max values of 5 to 10 mice per group from 5 independent experiments. Statistical analyses were performed using the Mann-Whitney test. \*\*P<0.01; \*\*\*P<0.001; \*\*\*\* P<0.0001.

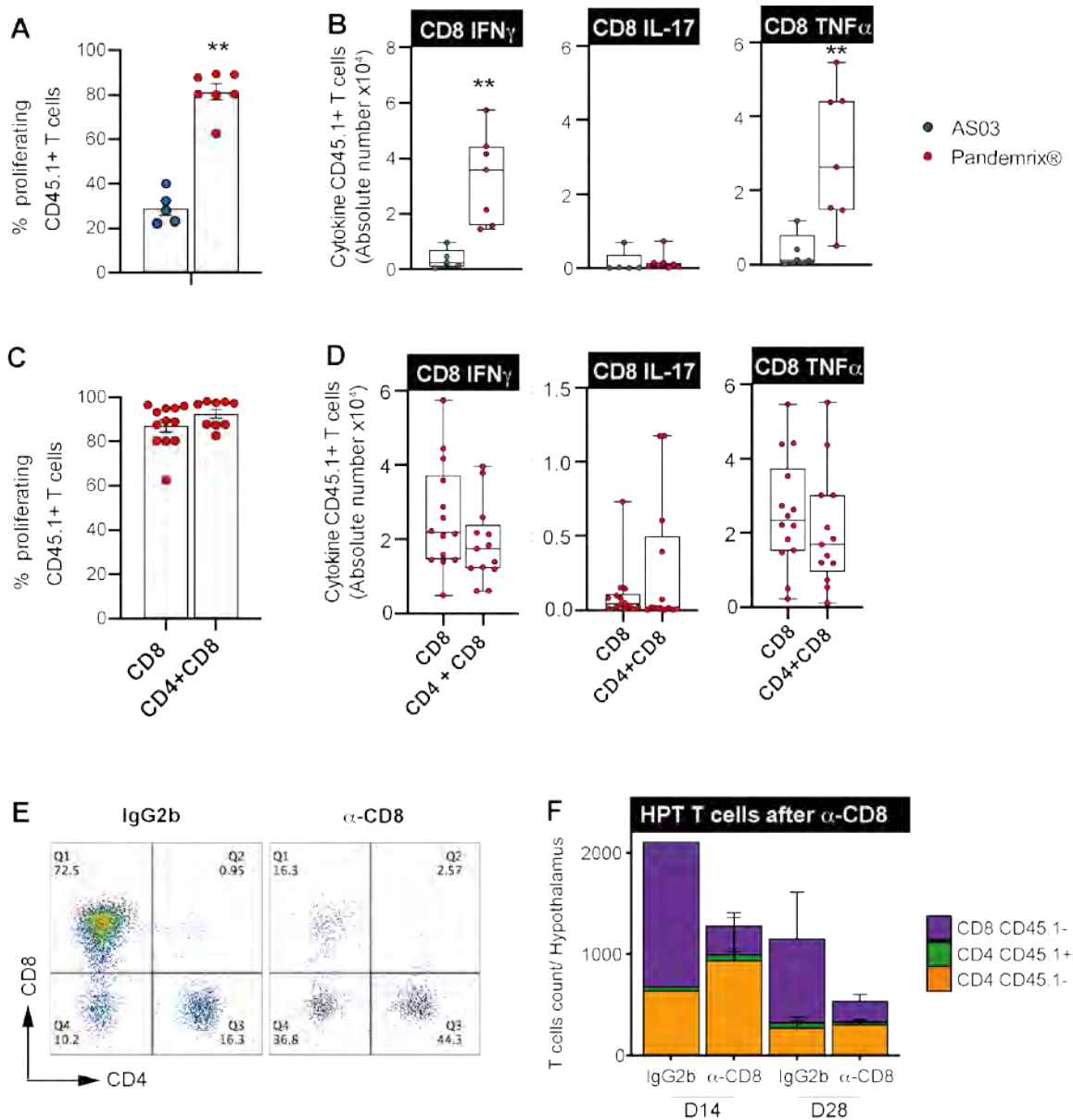


**Fig. S3. Analysis of T cell infiltration upon immunization in the hypothalamus and other CNS regions.** (A) Histological staining for CD3 (brown) in a representative coronal brain section of an Orex-HA animal 14 days after transfer of a combination of HA-specific CD4 T cells and HA-specific CD8 T cells and immunization with Pandemrix®. Scale bar represent 500  $\mu$ m. (B) CD3 T cell density in different brain regions (cortex, hippocampus, thalamus) of control (blue triangles) and Orex-HA (red circles) mice 14 days after transfer of HA-specific CD4 T cells and HA-specific CD8 T cells and immunization with Pandemrix®. Results are

expressed as mean  $\pm$  SEM of 9 to 14 mice per group. (C) CD3 T cell density in the hypothalamus of control (blue triangles) and Orex-HA (red circles) mice 14 days after transfer of HA-specific CD4 T cells and HA-specific CD8 T cells and immunization with Pandemrix<sup>®</sup>, AS03 adjuvant or saline. Results are expressed as mean  $\pm$  SEM of 2 to 16 mice per group. Results from the Pandemrix<sup>®</sup> vaccination groups are already depicted in Fig. 2B. (D) Representative histograms of MHC class II expression on microglial cells, macrophages and dendritic cells from the hypothalamus of Orex-HA mice after AS03 or Pandemrix<sup>®</sup> immunization. (E) Proportion of CD45<sup>dim</sup> CD11b<sup>+</sup> microglia expressing MHC class II molecules in the hypothalamus of Orex-HA mice after AS03 or Pandemrix<sup>®</sup> immunization. Statistical analyses were performed using the Mann-Whitney test. \*P < 0.05; \*\*\*\*P < 0.0001.



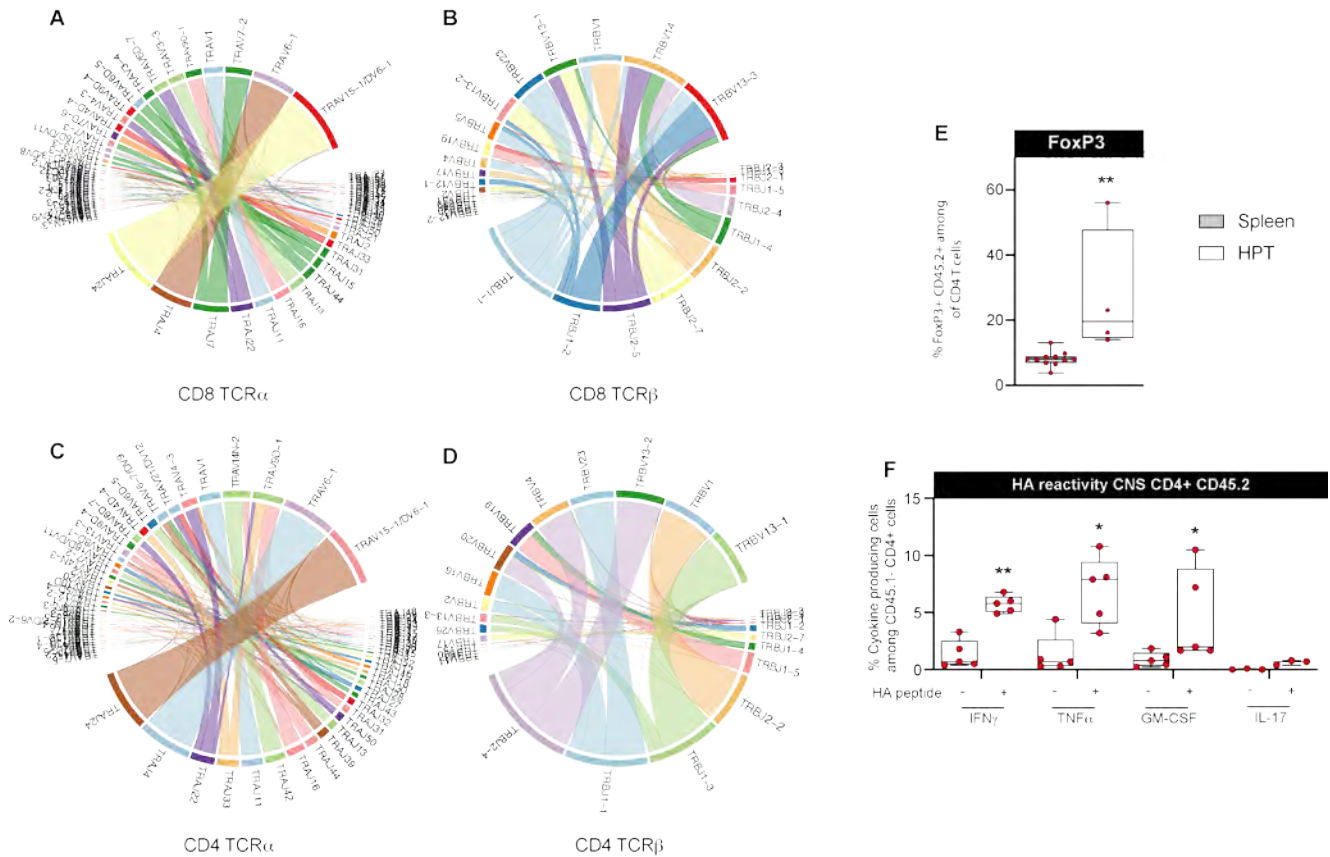
**Fig. S4. Gene expression profile of hypothalamus-infiltrating CD4 T cells compared to that of their peripheral counterparts.** (A) Schematic depiction of the experimental design followed for the RNAseq analysis. (B) Left, Top 20 over-representation analysis KEGG pathways generated from the differentially expressed genes (DEGs;  $p_{adj}$  cutoff < 0.05 and fold change > 1.5) between hypothalamus vs. spleen endogenous CD4 T cells. Right, Top 20 over-representation analysis KEGG pathways generated from the DEGs between hypothalamus vs. spleen transferred CD4 T cells. (C) Heatmap of genes of interest chosen from the top KEGG pathways.



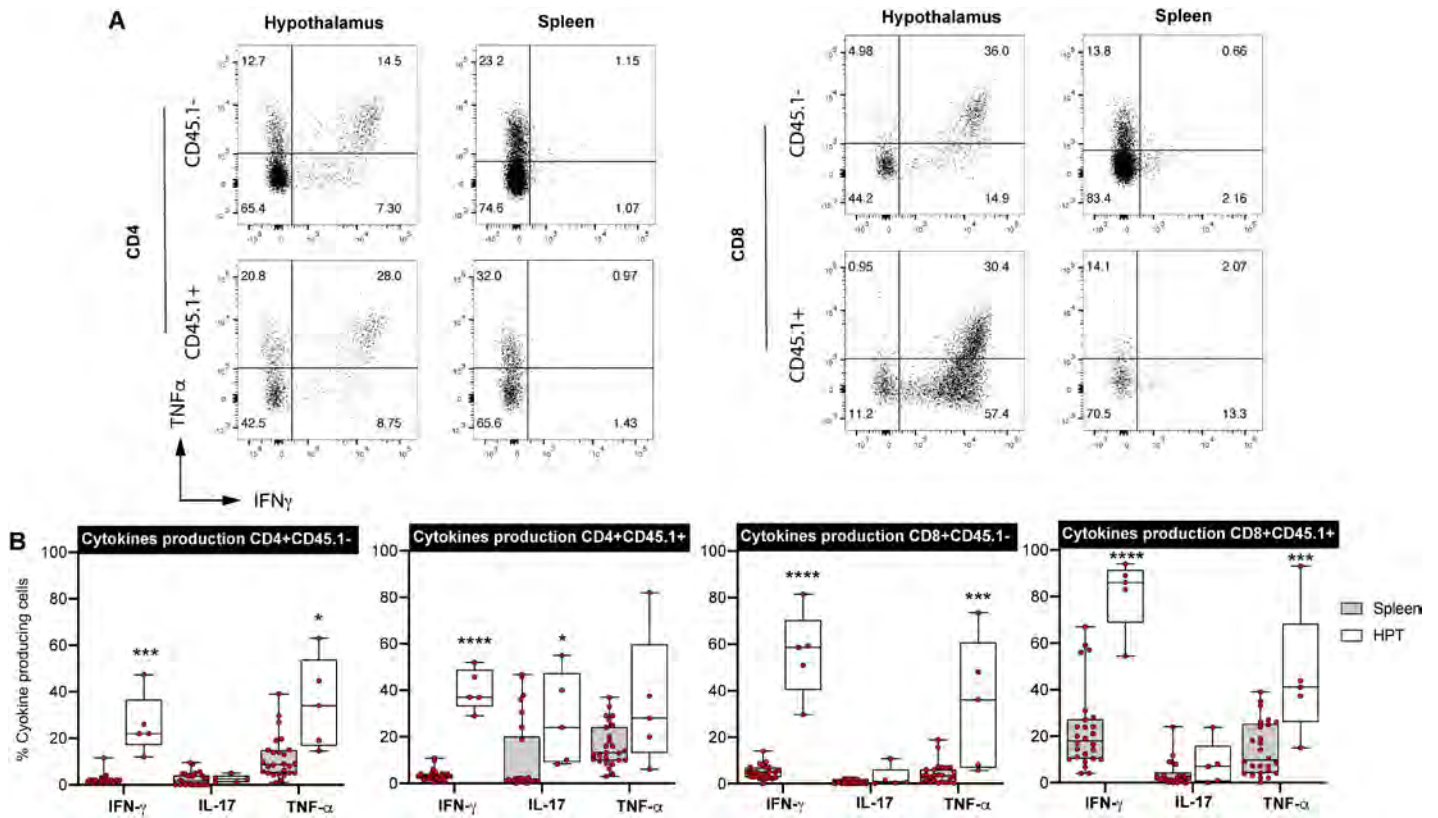
**Fig. S5. Efficient activation of CD8 T cells and impact of their depletion.** (A-B) Proliferation (A) and differentiation (B) of CD45.1+ HA-specific CD8 T cells following their transfer in recipient Orex-HA animals, in absence of co-transferred CD4 T cells, and immunization with Pandemrix® (red circles) or AS03 adjuvant (blue circles). Data come from the draining lymph nodes 5 days after immunization. Results are expressed as mean  $\pm$  SEM of 5 to 7 mice per group from 4 experiments. (C-D) Proliferation (C) and differentiation (D) of CD45.1+ HA-specific CD8 T cells following their transfer in recipient Orex-HA animals, in absence or presence of co-



transferred CD4 T cells, following immunization with Pandemrix® (red circles). Data originate from the draining lymph nodes 5 days after immunization. Results are expressed as mean  $\pm$  SEM of 9 to 14 mice per group from 3 experiments. (A and C) Proportion of CTV<sup>low</sup> CD45.1+ HA-specific CD8 T cells. The seven data points from the condition CD8 alone and vaccination with Pandemrix® shown in A are also used in C. (B and D) Absolute numbers of CD45.1+ HA-specific CD8 T cells producing IFN $\gamma$ , IL-17, and TNF $\alpha$ . Boxplots are depicting interquartile range and median, while whiskers extend between min and max values. (E) Representative FACS plot of the endogenous CD45.1-negative T cells infiltrating the hypothalamus 14 days after transfer of HA-specific CD4 T cells, Pandemrix® immunization and treatment with either control IgG2b (left) or anti-CD8 antibody (right). (F) Absolute numbers of hypothalamus-infiltrating T cells 14 and 28 days after transfer of HA-specific CD4 T cells, Pandemrix® immunization and treatment with either control IgG2b or anti-CD8 antibody. Statistical analyses were performed using the Mann-Whitney test. \*\*P < 0.01.



**Fig. S6. Diversity of endogenous T cells infiltrating the hypothalamus.** (A-D) Chord diagrams of Variable and Joining gene segment rearrangements for TCR $\alpha$  (A,C) and TCR $\beta$  (B,D) chains of CD45.1-negative endogenous CD8 (A, B) and CD4 (C, D) T cells infiltrating the hypothalamus of Orex-HA mice 14 days after vaccination. (E) Proportion of FoxP3<sup>+</sup> regulatory CD4 T cells among endogenous (CD45.1-) CD4 T cells in the spleen or hypothalamus of Orex-HA mice, at day 14 after transfer of both HA-specific CD4 and CD8 T cells and vaccination with Pandemrix<sup>®</sup>. Results are expressed as mean  $\pm$  SEM from individual spleens and 4 pools of 4 to 6 mice for hypothalamus-infiltrating T cells. Statistical analyses were performed using the Mann-Whitney test. (F) Frequency of endogenous CD45.1- CD4 T cells from the hypothalamus of Orex-HA mice producing IFN $\gamma$ , TNF $\alpha$ , GM-CSF and IL-17 with or without HA peptide stimulation. Analyses were performed by flow cytometry, 28 days after transfer of HA-specific CD4 and CD8 T cells and Pandemrix<sup>®</sup> immunization. Boxplots are depicting interquartile range and median, while whiskers extend between min and max values of 3 to 5 pools of 4 to 6 mice.



**Fig. S7. Cytokine production by hypothalamus-infiltrating T cells.** (A) Representative FACS plot of intracellular IFN $\gamma$  and TNF $\alpha$  expression, following PMA/ionomycin stimulation, by spleen and hypothalamus-infiltrating T cells of Orex-HA mice 14 days after HA-specific T cell transfer and Pandemrix<sup>®</sup> immunization. CD4 and CD8 T cells of endogenous (CD45.1-negative) or transferred (CD45.1-positive) origin are analyzed separately. (B) Quantification of production of IFN $\gamma$ , IL-17 and TNF $\alpha$  upon PMA/ionomycin stimulation by transferred CD45.1+ or endogenous CD45.1- CD4 and CD8 T cells from the hypothalamus (HPT) and spleen of Orex-HA mice 14 days after Pandemrix<sup>®</sup> immunization. Boxplots are depicting interquartile range and median, while whiskers extend between min and max values of 26 individual spleens and of 4 to 8 pooled hypothalami from 5 independent experiments. Statistical analyses were performed using the Mann-Whitney test. \*P < 0.05; \*\*\*P < 0.001; \*\*\*\*P < 0.0001.

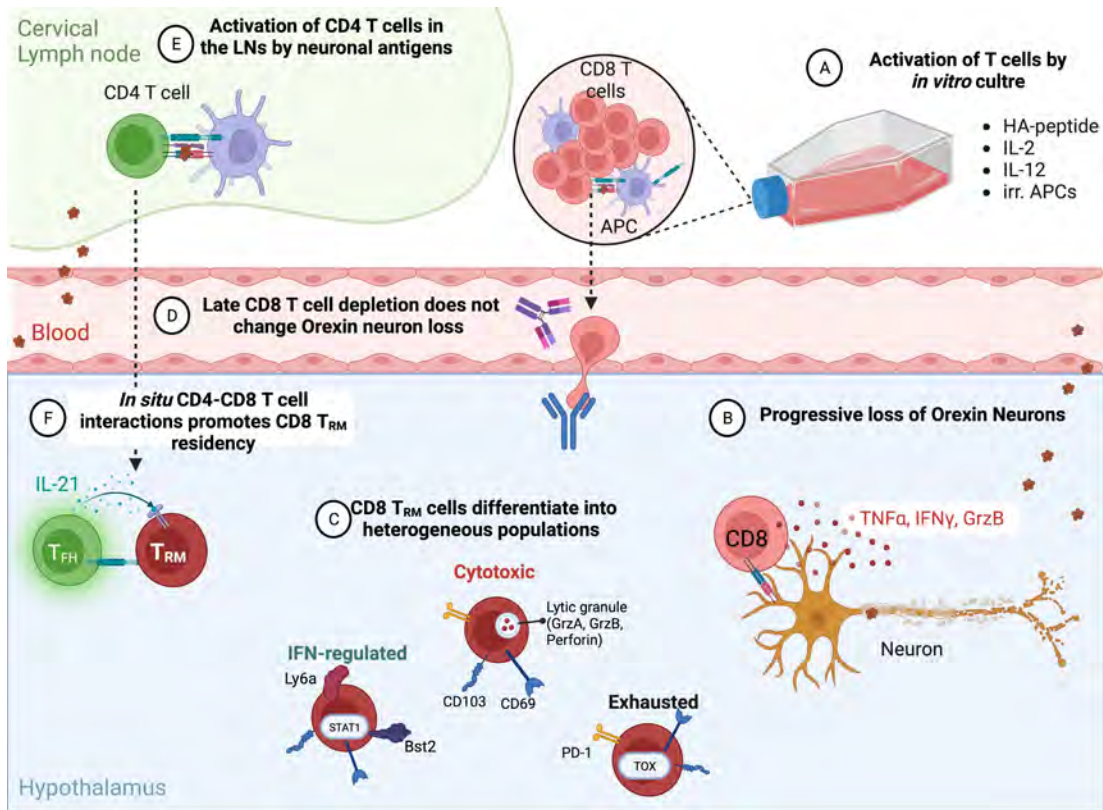
## 2.2 Paper: Tissue-resident CD8<sup>+</sup> T cells drive compartmentalized and chronic autoimmune damage against CNS neurons.

From our previous published work, we know that the destruction of Orexin-neurons is mediated by autoreactive CD8<sup>+</sup> T cells that are able to persist in the hypothalamus for at least two months (Bernard-Valnet et al. 2016). The phenotype of these persistent CD8<sup>+</sup> T cells had not yet been studied. It was very likely they possess a T<sub>RM</sub> signature and indeed our findings confirmed this hypothesis. Converging, the RNA sequencing analysis in the vaccine model of autoreactive, hypothalamic CD8 T cells revealed a CD8 T<sub>RM</sub> cell signature among them.

Although many studies about T<sub>RM</sub> cells were published in the past ten years, to date the function and diversity of this T memory lymphocytes subset remain poorly understood, especially their possible involvement in autoimmune diseases. This is important as the treatment of Natalizumab and Fingolimod is inefficient for secondary progressive MS patients (Naegelin et al. 2019). Following this idea, Fingolimod and Natalizumab treatment would only target circulating, but not tissue-resident, T cells. Therefore, T<sub>RM</sub> cells could be key drivers of chronic CNS autoimmune diseases. Nevertheless, in the autoimmune mouse model EAE, the treatment with Fingolimod during the chronic phase alleviated clinical scores and reduced the number of CD4 T cells in the CNS (Choi et al. 2011, Farez et al. 2016). Therefore, T cells in this specific model that both studies used seemed not to differentiate into T<sub>RM</sub> cells. Our models provide us therefore with a novel autoimmune mouse model to study the impact of a CD8 T<sub>RM</sub> cell population as key drivers of chronic damage to CNS neurons.

One goal of this study was to understand how to reverse neuronal damage through the depletion of CD8 T<sub>RM</sub> cells. This might be an interesting therapeutic target for NT1, but also other CNS autoimmune diseases. For relapsing diseases such as MS, vitiligo, and psoriasis, the selective depletion of T<sub>RM</sub> cells represents a crucial therapeutic target (Wu H et al. 2018), where systemic treatment does not modify the disease course. We developed several depletion strategies to reduce the number of CD8 T<sub>RM</sub> cells in the CNS and partially rescue the orexin neuron loss.

Finally, we provided evidence that in 10 PCD patients, CD8 T cells were found in the brain parenchyma and showed expression of several T<sub>RM</sub> markers that we identified in the mouse model, including CD69, CD103, PD-1, TOX, and Bcl-2. This is the first time that the phenotype of the CD8 T cells in the brain parenchyma of PCD patients has been more closely examined.



### Highlights

- Antigen-specific CD8 T cells differentiate in the CNS into  $T_{RM}$  cells and are found in **proximity to their neuronal target**.
- The neuronal destruction was **progressive** rather than abrupt.
- Anti-CD8 treatment does not rescue neuronal loss, suggesting **CD8  $T_{RM}$  cells are key drivers of CNS damage**.
- scRNA sequencing revealed three heterogeneous CD8  $T_{RM}$  populations, expressing high levels of **cytotoxic molecules, exhaustion-regulated** and **IFN-regulated** genes.
- **CD4  $T_{FH}$  cells** support the persistence of CD8  $T_{RM}$  cells in the hypothalamus.

## AUTOIMMUNE DISEASES

Tissue-resident CD8<sup>+</sup> T cells drive compartmentalized and chronic autoimmune damage against CNS neurons

David Frieser<sup>1</sup>, Aurora Pignata<sup>1</sup>, Leila Khajavi<sup>1</sup>, Danielle Shlesinger<sup>2</sup>, Carmen Gonzalez-Fierro<sup>1</sup>, Xuan-Hung Nguyen<sup>1</sup>, Alexander Yermanos<sup>2,3,4</sup>, Doron Merkler<sup>4,5</sup>, Romana Höftberger<sup>6</sup>, Virginie Desestret<sup>7</sup>, Katharina M. Mair<sup>8</sup>, Jan Bauer<sup>8</sup>, Frederick Masson<sup>1\*†</sup>, Roland S. Liblau<sup>1,9\*†</sup>

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The mechanisms underlying the chronicity of autoimmune diseases of the central nervous system (CNS) are largely unknown. In particular, it is unclear whether tissue-resident memory T cells ( $T_{RM}$ ) contribute to lesion pathogenesis during chronic CNS autoimmunity. Here, we observed that a high frequency of brain-infiltrating CD8<sup>+</sup> T cells exhibit a  $T_{RM}$ -like phenotype in human autoimmune encephalitis. Using mouse models of neuronal autoimmunity and a combination of T single-cell transcriptomics, high-dimensional flow cytometry, and histopathology, we found that pathogenic CD8<sup>+</sup> T cells behind the blood-brain barrier adopt a characteristic  $T_{RM}$  differentiation program, and we revealed their phenotypic and functional heterogeneity. In the diseased CNS, autoreactive tissue-resident CD8<sup>+</sup> T cells sustained focal neuroinflammation and progressive loss of neurons, independently of recirculating CD8<sup>+</sup> T cells. Consistently, a large fraction of autoreactive tissue-resident CD8<sup>+</sup> T cells exhibited proliferative potential as well as proinflammatory and cytotoxic properties. Persistence of tissue-resident CD8<sup>+</sup> T cells in the CNS and their functional output, but not their initial differentiation, were crucially dependent on CD4<sup>+</sup> T cells. Collectively, our results point to tissue-resident CD8<sup>+</sup> T cells as essential drivers of chronic CNS autoimmunity and suggest that therapies targeting this compartmentalized autoreactive T cell subset might be effective for treating CNS autoimmune diseases.

## INTRODUCTION

Chronic autoimmune diseases of the central nervous system (CNS) can target different CNS-resident cell types, including oligodendrocytes as suspected in multiple sclerosis (MS) (1, 2), astrocytes as identified in neuromyelitis optica spectrum disorder (3), or neurons in conditions such as autoimmune encephalitis, narcolepsy, or autoimmune paraneoplastic syndromes (4, 5). An autoimmune process also appears at play in prevalent neurodegenerative disorders such as Parkinson's disease (6). In most CNS autoimmune diseases, the precise immune mechanisms propagating tissue damage and the resulting clinical progression remain elusive. Chronic progressive or waxing and waning inflammation is, nevertheless, a pathological hallmark of many CNS autoimmune conditions.

There is evidence that antigenic persistence is a key parameter for chronic inflammation, and complete destruction of the cellular targets often results in subsiding of local inflammation (5, 7, 8). However, this denouement may take months or years or cannot even be reached when the cells targeted by the autoimmune process regenerate. Continuous or repeated waves of incoming lymphocytes may contribute to the chronicity of the CNS autoimmune process.

Therapies inhibiting immune cell transmigration into the CNS have reached the clinic, most notably in MS. However, therapeutic approaches targeting immune cell diapedesis have failed in progressive MS, suggesting an uncoupling of the chronic destructive autoimmune process from systemic immune responses (9). In chronic inflammatory CNS diseases, a compartmentalized immune response develops, encompassing CNS-resident T cells, B cells, and myeloid cells, which is associated with progressive tissue damage (10–12). Therefore, better understanding the cellular actors and mechanisms underlying the CNS-restricted immune response would be essential for the development of tailored therapeutic approaches, in particular in chronic disease stages.

Tissue-resident memory T cells ( $T_{RM}$ ) have been initially described in the context of infectious diseases, as a population of long-lived memory T cells remaining in barrier tissues after the resolution of the initial infection (13, 14). Upon entry within the tissues,  $T_{RM}$  undergo a transcriptional program of differentiation, facilitating long-term tissue residency. This program is controlled by the transcription factors Hobit and Blimp1, which inhibit expression of proteins, including KLF2, S1PR1, CCR7, and CD62L, involved in T cell tissue egress and/or homing and retention in lymphoid organs (15). Conversely,  $T_{RM}$  are characterized by surface markers, such as CD69, CD103, CXCR3, CXCR6, and CD49A, facilitating their retention within nonlymphoid tissues. Upon tissue reinfection,  $T_{RM}$  proliferate and exhibit cytotoxic and inflammatory properties, culminating in a tissue-specific anti-infectious response (13, 14).

The presence of  $T_{RM}$  has now been documented in tissues other than barrier tissues, such as the CNS (16, 17), and in conditions as diverse as cancer, graft-versus-host disease, allergy, and autoimmunity (13, 18–21). In chronic inflammatory/autoimmune diseases, such as asthma, psoriasis, vitiligo, inflammatory bowel disease, and type 1 diabetes,  $T_{RM}$  have been identified in the affected tissue in animal models and/or human conditions (19, 21, 22). In MS, CD8<sup>+</sup>

<sup>1</sup>Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), University of Toulouse, CNRS, INSERM, UPS, 31024 Toulouse, France. <sup>2</sup>Institute of Microbiology, ETH Zurich, 8093 Zurich, Switzerland. <sup>3</sup>Department of Biosystems Science and Engineering, ETH Zurich, 4058 Basel, Switzerland. <sup>4</sup>Department of Pathology and Immunology, University of Geneva, 1211 Geneva, Switzerland. <sup>5</sup>Division of Clinical Pathology, Geneva University Hospital, 1211 Geneva, Switzerland. <sup>6</sup>Division of Neuropathology and Neurochemistry, Department of Neurology, Medical University of Vienna, 1090 Vienna, Austria. <sup>7</sup>National Reference Center for Paraneoplastic Neurological Syndromes, MeLiS-UCBL-CNRS, INSERM, Hôpital Neurologique, Hospices Civils de Lyon, 69500 Lyon, France. <sup>8</sup>Center for Brain Research, Medical University of Vienna, 1090 Vienna, Austria. <sup>9</sup>Department of Immunology, Toulouse University Hospital, 31300 Toulouse, France.

\*Corresponding author. Email: frederick.masson@inserm.fr (F.M.); roland.liblau@inserm.fr (R.S.L.)

†These authors contributed equally to this work.

T cells expressing CD69 and CD103 represent a sizeable proportion of CNS-infiltrating T cells (10, 23). Recent data even suggest that  $T_{RM}$  can partake in the pathologic intrathecal immune reaction early in the disease process (24). Determining the role of  $T_{RM}$  in promoting chronic autoimmune CNS tissue damage is, therefore, an important challenge given their long-lasting inflammatory potential.

We identified a large fraction of  $CD8^+$  T cells expressing CD103 in human brain samples from patients suffering from autoimmune diseases targeting neurons. To get further insights on the role and heterogeneity of tissue-resident  $CD8^+$  T cells and their functional interactions with other immune cell subsets in the context of autoimmune CNS tissue damage, we devised mouse models of neuron-targeting chronic autoimmunity and made use of single-cell transcriptomics, histology, and high-dimensional flow cytometric analyses. Our data strongly suggest that tissue-resident  $CD8^+$  T cells play a central role in the progressive autoimmune-mediated damage of CNS neurons.

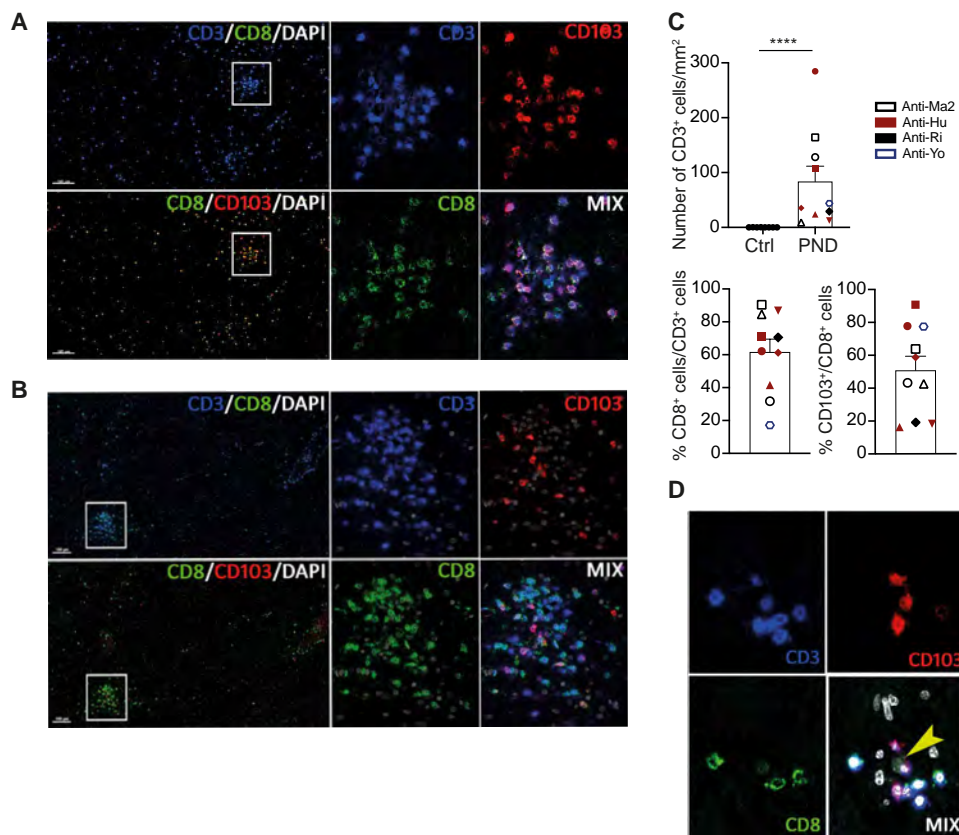
## RESULTS

### $CD8^+$ $T_{RM}$ -like cells are detected within lesions of patients with neuron-targeting autoimmune encephalitis

To assess whether T cells expressing prototypic markers of  $T_{RM}$  cells could be detected in CNS autoimmune diseases, we performed multiplexed immunofluorescence analysis for CD3, CD8, and CD103 in lesions from 10 patients with well-defined encephalomyelitis. We selected samples from four distinct conditions associated with autoantibodies directed against intracellular neuronal self-antigens (table S1). In these conditions,  $CD8^+$  T cells are likely responsible for neuronal damage, which can be widespread or very selective (8, 25). All samples were characterized by prominent T cell infiltrations within the brain parenchyma (and one dorsal root ganglion) as compared with control samples, although the T cell density varied depending on the region and the patient (Fig. 1, A to C). On average, 60% of T cells were  $CD8^+$  T cells, and among them, 50% expressed CD103 (Fig. 1C).  $CD103$ -expressing  $CD8^+$  T cells were dispersed in the brain parenchyma and, at places, formed aggregates (Fig. 1, A and B). Some  $CD103^+$  T cells were found in close proximity to neurons (Fig. 1D). The high proportion of CNS-infiltrating  $T_{RM}$ -like  $CD8^+$  T cells identified in human samples prompted us to interrogate their pathogenic relevance using mouse models targeting a discrete population of CNS neurons.

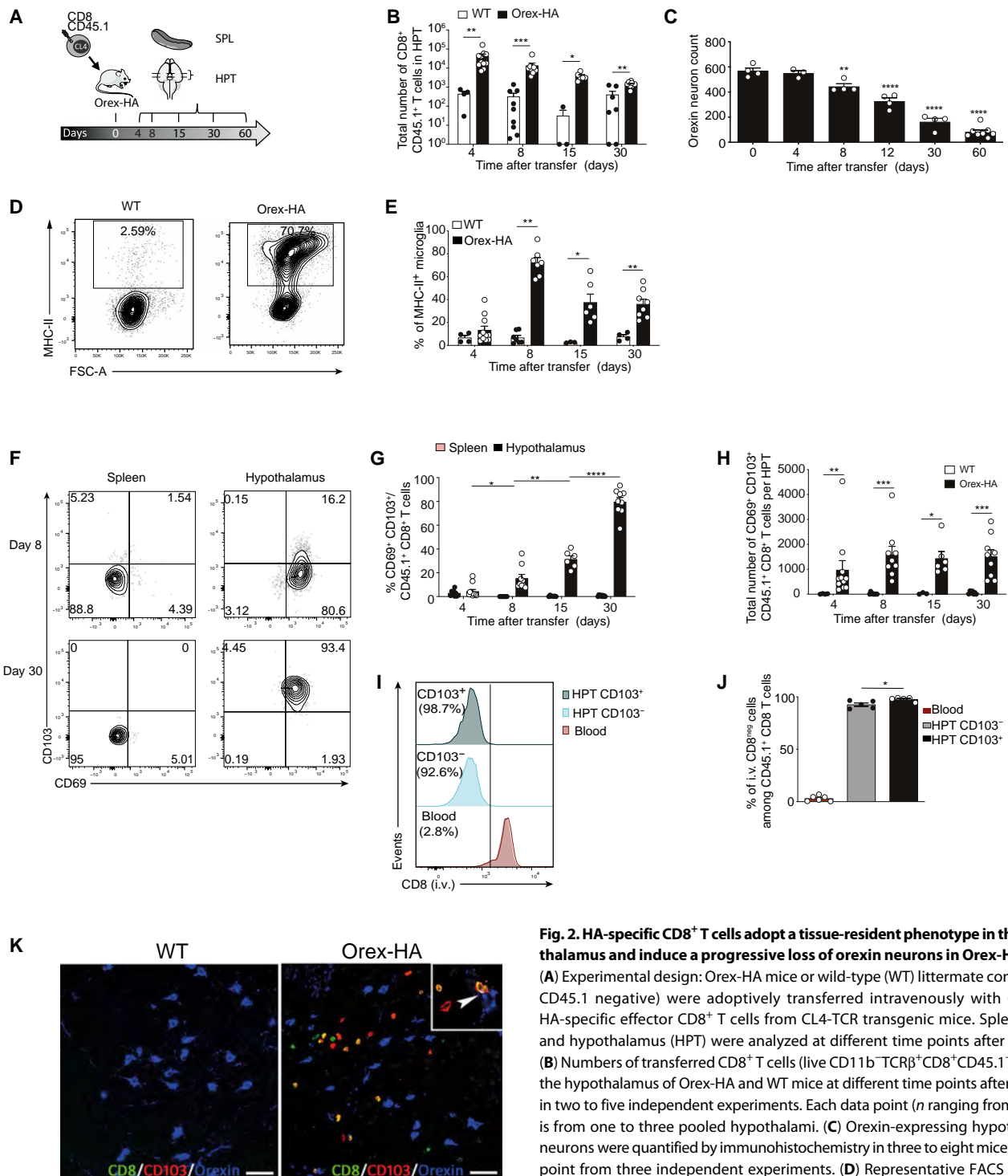
### Temporal association between tissue-resident $CD8^+$ T cell differentiation and progressive neuron loss in experimental CNS autoimmunity

We previously generated the Orex-HA mouse model in which orexin-producing neurons residing in the hypothalamus selectively express a model self-antigen, namely, hemagglutinin (HA), from the H1N1 influenza virus (26). Upon adoptive transfer of activated ( $CD45.1^+$ ) HA-specific  $CD8^+$  T cells (Fig. 2A), we analyzed by flow cytometry these cells from the dissected hypothalami of Orex-HA or littermate control mice over time. The number of HA-specific  $CD8^+$  T cells within the hypothalamus peaked at day 4 after transfer, contracted at day 8, but remained relatively stable from day 15 onward (Fig. 2B). We next asked whether this T cell persistence within the hypothalamus was associated with a progressive loss of orexinergic neurons and localized inflammation. To this end, orexinergic neuron counts within the hypothalamus of Orex-HA mice were assessed over time by immunohistochemistry. Orexin<sup>+</sup> neurons were progressively lost after adoptive transfer, reaching about



**Fig. 1.  $CD8^+$  T cells are largely represented within lesions from neuron-targeting autoimmune encephalitis.**

$T_{RM}$  (A) Representative immunostaining of  $CD103$ -expressing  $CD8^+$   $T_{RM}$ -like cells within brainstem lesions of anti-Hu antibody-associated paraneoplastic encephalomyelitis and (B) anti-Ri antibody-associated encephalitis. Scale bars, 100  $\mu$ m. (C) Quantification of the density of T cells (top), the proportion of  $CD8^+$  cells among T cells (bottom left), and the frequency of  $CD103$ -expressing  $CD8^+$  T cells (bottom right) in brains, and one dorsal root ganglion, of 10 patients with autoimmune diseases targeting intracellular neuronal self-antigens (five anti-Hu, three anti-Ma2, one anti-Yo, and one anti-Ri antibody-associated syndromes) and 8 nonneurological controls. Each symbol represents a given patient, and the color indicates the antibody-associated syndrome. Statistical analyses were performed using the Mann-Whitney test. (D) Microphotographs documenting close apposition of  $CD3^+CD8^+CD103^+$  and  $CD3^+CD8^+CD103^+$  T cells to a neuron (arrowhead) in the cerebellum of anti-Ma2 antibody-associated encephalitis. DAPI, 4',6-diamidino-2-phenylindole. \*\*\*\* $P < 0.0001$ .



**Fig. 2. HA-specific CD8<sup>+</sup> T cells adopt a tissue-resident phenotype in the hypothalamus and induce a progressive loss of orexin neurons in Orex-HA mice.**

(A) Experimental design: Orex-HA mice or wild-type (WT) littermate controls (all CD45.1 negative) were adoptively transferred intravenously with CD45.1<sup>+</sup> HA-specific effector CD8<sup>+</sup> T cells from CL4-TCR transgenic mice. Spleen (SPL) and hypothalamus (HPT) were analyzed at different time points after transfer. (B) Numbers of transferred CD8<sup>+</sup> T cells (live CD11b<sup>-</sup>TCRβ<sup>+</sup>CD8<sup>+</sup>CD45.1<sup>+</sup> cells) in the hypothalamus of Orex-HA and WT mice at different time points after transfer in two to five independent experiments. Each data point (*n* ranging from 3 to 11) is from one to three pooled hypothalami. (C) Orexin-expressing hypothalamic neurons were quantified by immunohistochemistry in three to eight mice per time point from three independent experiments. (D) Representative FACS plots on day 8 after transfer; FSC-A, Forward-side scatter area, and (E) quantification of MHC-II expression on microglia (live CD45.2<sup>int</sup>CD11b<sup>int</sup>Ly-6c<sup>-</sup> cells) in the hypothalamus of Orex-HA versus WT mice in two (day 4) to three (days 8 to 30) independent experiments. Each data point (*n* = 3 to 11) is from one to three pooled hypothalami. (F) Representative FACS plots and (G) proportion of transferred CD8<sup>+</sup> T cells expressing CD69 and CD103 in the spleen and hypothalamus of Orex-HA mice at different time points after transfer. (H) Numbers of CD69<sup>+</sup>CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA and WT mice in two (day 4), three (days 8 to 15), and five (day 30) independent experiments. Each data point (*n* = 3 to 11) is from one to three pooled hypothalami. (I) Representative histograms and (J) frequency of transferred CD8<sup>+</sup> T cells, from hypothalamus or blood that were not labeled by fluorescent anti-CD8 mAb injected intravenously (i.v.) 5 min before euthanasia of Orex-HA mice at day 30 after transfer, according to their CD103 expression. Data are from three independent experiments with one to three pooled Orex-HA mice per data point (*n* = 5 to 6). (K) Immunostaining of hypothalami of WT and Orex-HA mice at day 30. The inset shows a CD103<sup>+</sup>CD8<sup>+</sup> T cell in close apposition to an orexin-expressing neuron. Scale bars, 25 μm. Statistical analyses were performed using multiple Mann-Whitney test with Benjamini-Hochberg correction (B, E, and H), one-way ANOVA with Dunnett or Tukey's post hoc test (C and G), and paired Student's *t* test (J). \**P* < 0.05, \*\*\**P* < 0.01, \*\*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

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a 70% reduction by day 30 (Fig. 2C). This loss was associated with an increased major histocompatibility complex class II (MHC-II) expression on microglial cells (Fig. 2, D and E) in the dissected hypothalami.

These data indicate that an inflammatory process persisted long after the initial seeding of the antigen-specific CD8<sup>+</sup> T cells within the hypothalamus and was associated with the progressive loss of orexinergic neurons. This could be the result of the continuous recruitment of transferred antigen-specific CD8<sup>+</sup> T cells from the circulation to the hypothalamus and/or their stable residence within the CNS microenvironment, a hallmark of T<sub>RM</sub>. To decipher whether HA-specific CD8<sup>+</sup> T cells could differentiate into tissue-resident CD8<sup>+</sup> T cells during CNS autoimmunity, we phenotypically characterized these cells before and after transfer into Orex-HA mice. Before their transfer, activated HA-specific CD8<sup>+</sup> T cells did not express CD103 (fig. S1A). The hypothalamic HA-specific CD8<sup>+</sup> T cells started to coexpress the canonical T<sub>RM</sub> markers CD103 and CD69 by day 8 after transfer, and the proportion of T<sub>RM</sub>-like cells progressively increased from 15.3% at day 8 to 78.5% at day 30 (Fig. 2, F and G). The absolute number of T<sub>RM</sub>-like cells in the hypothalamus remained stable from day 8 to day 30 (Fig. 2H). Local antigen stimulation appeared to be necessary to mobilize HA-specific tissue-resident CD8<sup>+</sup> T cells into the hypothalamus as none was detected in the spleen of Orex-HA mice (Fig. 2, F and G). Moreover, significantly fewer ( $P < 0.001$ ) tissue-resident CD8<sup>+</sup> T cells were found in the cerebellum, a brain region devoid of HA-expressing orexin neurons, and few, if any, were detected in the hypothalamus of wild-type recipient mice (Fig. 2H and fig. S1B). We confirmed the parenchymal location of the hypothalamic tissue-resident CD8<sup>+</sup> T cells by injecting mice intravenously with a fluorescently labeled anti-CD8 antibody that stained endovascular CD8<sup>+</sup> T cells before collecting the brain (Fig. 2, I and J). Immunohistochemistry confirmed the presence of CD103<sup>+</sup> CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice and revealed instances of close apposition between T<sub>RM</sub>-like cells and orexinergic neurons (Fig. 2K).

Collectively, the data revealed local differentiation of antigen-specific CD8<sup>+</sup> T cells expressing T<sub>RM</sub> markers during experimental CNS autoimmunity. These T cells were stably present within the hypothalamus, and their presence correlated with progressive loss of neurons and sustained CNS inflammation.

### Transcriptional signature of the autoreactive brain tissue-resident CD8<sup>+</sup> T cells

To further assess whether hypothalamic CD8<sup>+</sup> T cells were bona fide tissue-resident cells and to investigate their role in the pathogenic mechanisms, we performed a single-cell transcriptomic analysis [single-cell RNA sequencing (scRNA-seq)] of HA-specific CD8<sup>+</sup> T cells infiltrating the hypothalamus and spleen at day 30 after T cell transfer. The hypothalamic HA-specific CD8<sup>+</sup> T cells segregated away from their splenic counterparts on the Uniform Manifold Approximation and Projection (UMAP) representation (Fig. 3A). Genes differentially up-regulated in the T cells from the hypothalamus compared with the spleen were enriched in T<sub>RM</sub> markers (*Itgae*, *Itga1*, *Cxcr6*, *Bhlhe40*, *Runx3*, *Junb*, and *Rgs1*) and effector function associated genes (*Gzmb*, *Gzma*, *Ccl4*, *Xcl1*, and *Ifng*) (Fig. 3, B and D). Conversely, genes down-regulated in the hypothalamus-infiltrating CD8<sup>+</sup> T cells were involved in tissue egress (*S1pr1* and *Klf2*) and central memory T cells (*Tcf7*, *Sell*, and *Ccr7*) (Fig. 3, B and D). Gene set enrichment analysis (GSEA) further confirmed that the transcriptome of HA-specific hypothalamic CD8<sup>+</sup> T cells in comparison with their

splenic counterparts is strongly enriched for a previously published brain T<sub>RM</sub> gene signature (Fig. 3C) (27).

In addition, we validated the transcriptomic data by flow cytometry. The expression of T<sub>RM</sub>-associated markers CXCR6, CXCR3, and CD49A (encoded by *Itga1*) was increased in hypothalamic HA-specific CD8<sup>+</sup> T cells compared with their splenic counterparts at day 30 after transfer (fig. S2). Conversely, Ly-6c expression associated with vascular/recirculating T cells was decreased in hypothalamus-infiltrating T cells similarly to the transcription factors T-BET and EOMES (fig. S2), which interfere with T<sub>RM</sub> development. Moreover, analysis of GRANZYME B (Fig. 3E), interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor (TNF) (Fig. 3, F and G) expression confirmed the high effector potential of hypothalamic HA-specific tissue-resident CD8<sup>+</sup> T cells. As local cognate antigen expression has been shown to be an important mechanism of T<sub>RM</sub> maintenance within the tissues (28, 29), we assessed whether HA-specific tissue-resident CD8<sup>+</sup> T cells were proliferating locally. In line with a potential reactivation of HA-specific tissue-resident CD8<sup>+</sup> T cells within the hypothalamus, on average, 19.4% of these hypothalamic T cells expressed the proliferation marker KI-67 at day 30, whereas splenic HA-specific T cells were almost invariably negative, and only 3.6% of hypothalamic host CD8<sup>+</sup> T cells expressed KI-67 (Fig. 3H).

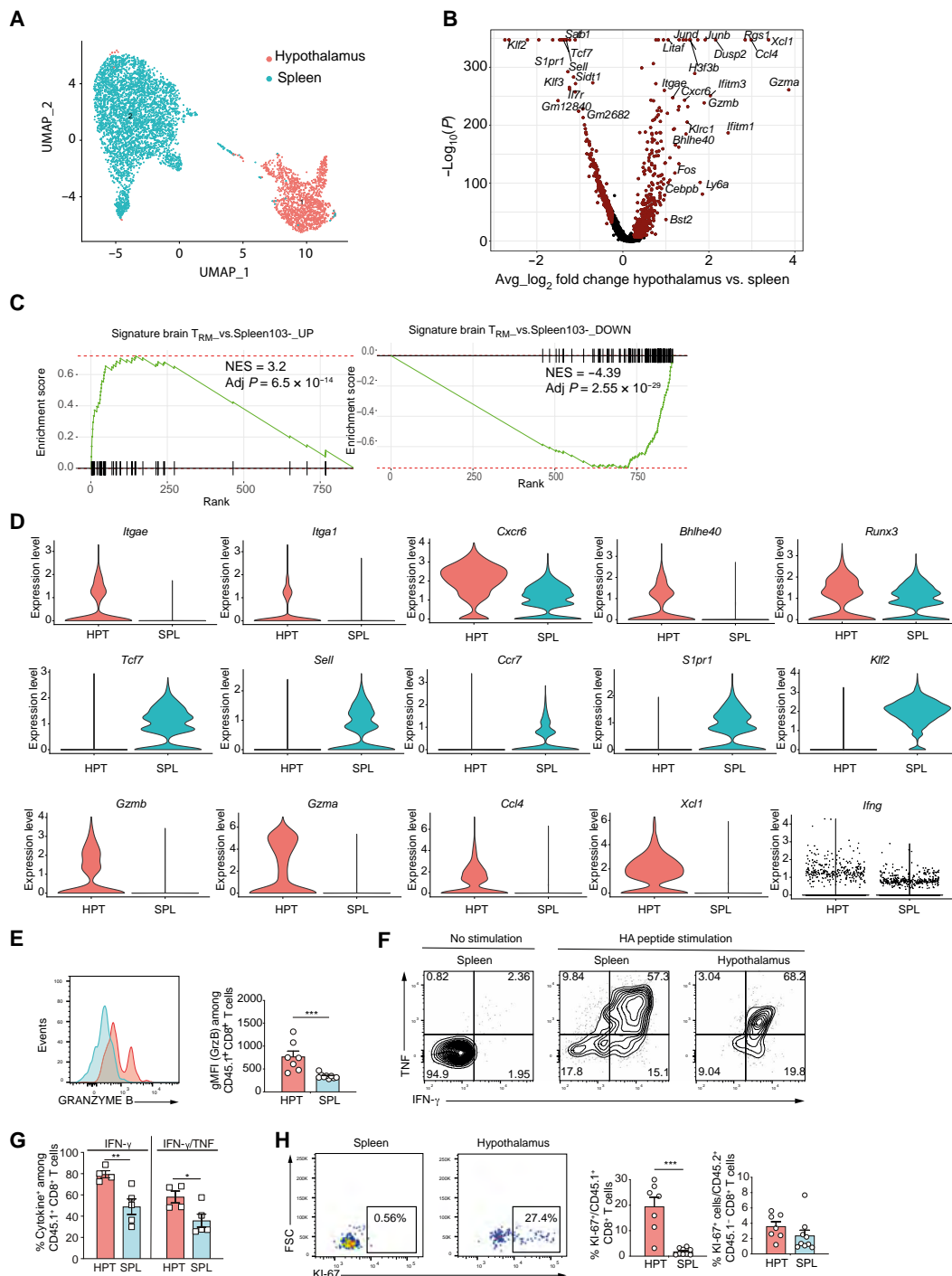
Together, these results confirmed the T<sub>RM</sub> differentiation status of hypothalamus-infiltrating HA-specific CD8<sup>+</sup> T cells and demonstrated that these cells proliferated and exhibited an effector phenotype within the brain microenvironment.

### Tissue-resident CD8<sup>+</sup> T cells infiltrating the hypothalamus are phenotypically and functionally heterogeneous

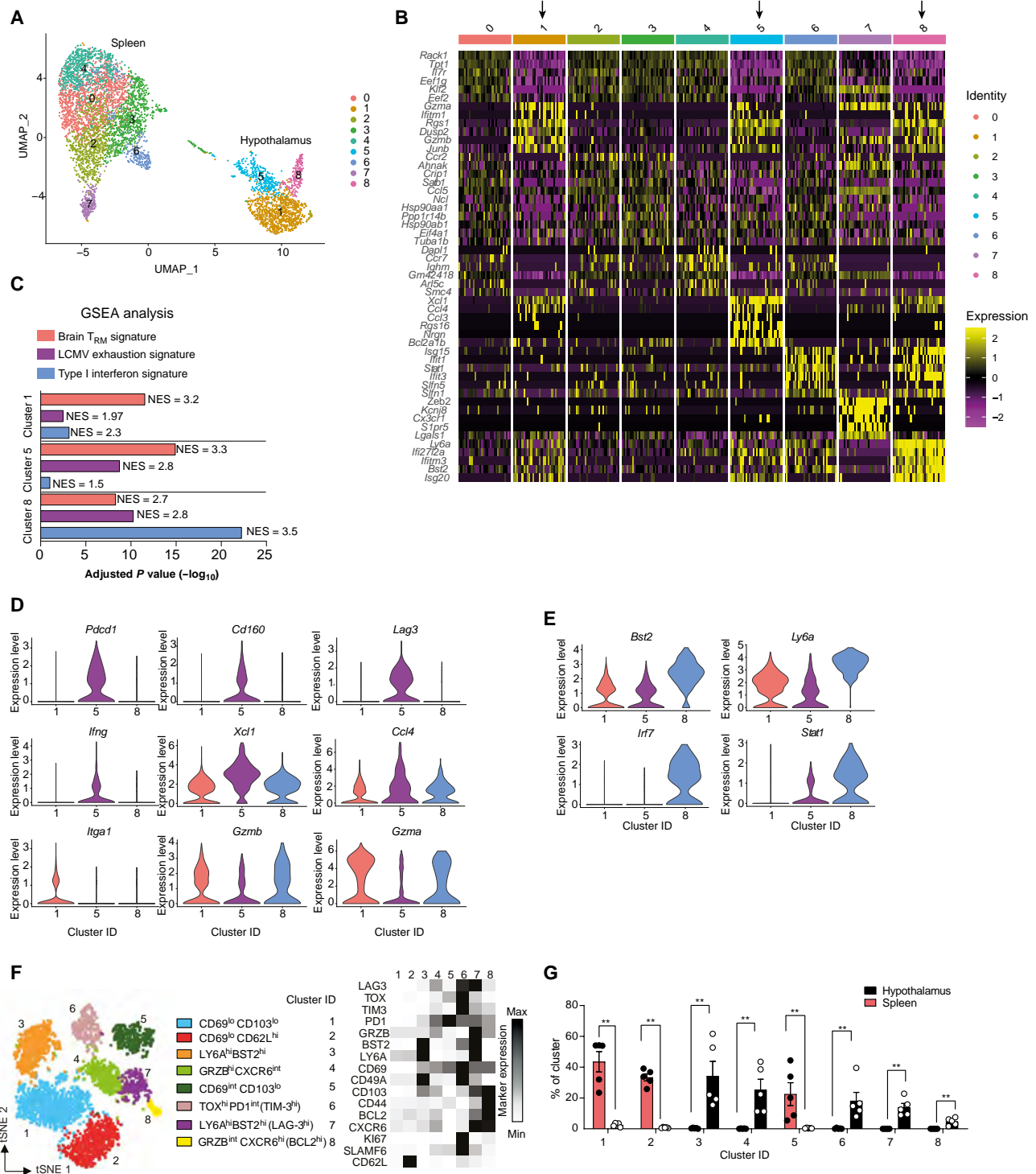
Next, we asked whether the tissue-resident CD8<sup>+</sup> T cells represented a homogeneous population or rather encompassed different subsets exhibiting different functions. Clustering analysis of the scRNA-seq data uncovered nine different clusters in HA-specific CD8<sup>+</sup> T cells isolated from the spleen and hypothalamus at day 30 after transfer, including three clusters, namely, 1, 5, and 8, specific to the hypothalamus (Fig. 4, A and B, and fig. S3A). A GSEA showed that all three hypothalamic clusters exhibited a strong enrichment in brain T<sub>RM</sub> gene signature (Fig. 4C and fig. S3, B and C).

As chronic antigenic stimulation could trigger an exhaustion program of differentiation, we then evaluated whether the three hypothalamic clusters exhibited such signature. The GSEA revealed that clusters 5 and 8 were also enriched for an exhausted T cell signature (Fig. 4C and fig. S3B). However, cluster 5 distinctively up-regulated coinhibitory receptors classically associated with exhausted T cells such as *Pdcd1*, *CD160*, *Lag3* (Fig. 4D), and *Havcr2*. By contrast, the exhaustion gene signature of cluster 8 appeared linked to the type I IFN signaling pathway (*Igs20*, *Ifi1*, *Ifi3*, *Ifi3b*, *Ifitm3*, *Ifi203*, and *Ifi2712a*). Accordingly, GSEA for IFN gene signature of the three clusters confirmed that differentially expressed genes in cluster 8 were strongly enriched in type I IFN gene signaling including *Bst2*, *Ly6a*, *Irf7*, and *Stat1* (Fig. 4, C and E). Clusters 1 and 8 were characterized by cells with the highest expression of cytotoxic molecules (*Gzma* and *Gzmb*). By contrast, cells from cluster 5 had a lower cytotoxic potential, but the highest cytokine and chemokine expression (*Ifng*, *Xcl1*, and *Ccl4*) despite the expression of exhaustion markers (Fig. 4D).

To further validate these three clusters, we analyzed expression of distinctive surface markers on hypothalamic and splenic HA-specific T cells by high-dimensional flow cytometry at day 30 after transfer.



**Fig. 3. The transcriptional analysis of autoreactive CD8<sup>+</sup> T cells from the hypothalamus of Orex-HA mice reveals their identity.**  $T_{RM}$  (A) UMAP representation of the scRNA-seq dataset from transferred HA-specific CD8<sup>+</sup> T cells from the spleen (blue) and hypothalamus (red) of 13 pooled Orex-HA mice on day 30. (B) Volcano plot of the differentially expressed genes between the hypothalamus and spleen, according to their adjusted  $P$  value and  $\log_2$  fold change (Log2FC). (C) Gene set enrichment analysis (GSEA) of a published brain  $T_{RM}$  signature in a ranked list of genes differentially expressed between hypothalamic compared with splenic transferred CD8<sup>+</sup> T cells of Orex-HA mice. NES, normalized enrichment score. (D) Violin plots of highly up- or down-regulated genes in HA-specific CD8<sup>+</sup> T cells from hypothalamus versus spleen. (E) Representative histogram and quantification of GRANZYME B expression [geometric mean fluorescence intensity (gMFI)] among splenic and hypothalamic transferred CD8<sup>+</sup> T cells from Orex-HA mice, on day 30, in three independent experiments. Each data point ( $n = 7$  to  $9$ ) involves two to six pooled mice. (F) Representative FACS plots and (G) frequency of TNF/IFN- $\gamma$  expression in splenic and hypothalamic transferred CD8<sup>+</sup> T cells from Orex-HA mice on day 30, 6 hours after ex vivo stimulation with the HA<sub>512-520</sub> peptide in two independent experiments. Each data point ( $n = 4$  to  $5$ ) is from three to six pooled mice. (H) Representative FACS plots and frequency of KI-67-expressing splenic and hypothalamic transferred or host CD8<sup>+</sup> T cells on day 30 in three independent experiments. Each data point ( $n = 7$  to  $9$ ) is from two to six pooled Orex-HA mice. Statistical analyses were performed using Mann-Whitney test (E and H) and two-way ANOVA with Sidak's post hoc test (G). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



**Fig. 4. Heterogeneity of autoreactive tissue-resident CD8<sup>+</sup> T cells in the hypothalamus.** (A) UMAP representation of SEURAT-generated clusters based on the scRNA-seq of splenic and hypothalamic transferred CD8<sup>+</sup> T cells from Orex-HA mice on day 30. (B) Heatmap of the five most highly expressed genes in the nine respective clusters. Arrows indicate the three hypothalamic clusters. (C) Bar graphs show the adjusted *P* value and normalized enrichment score (NES) for the indicated published gene signatures in the transcriptome of the three hypothalamic CD8<sup>+</sup> T cell clusters. LCMV, lymphocytic choriomeningitis virus. (D) Violin plots of the expression of selected genes from the effector and exhaustion signatures, and (E) type I interferon signature in the three hypothalamic tissue-resident CD8<sup>+</sup> T cell clusters. (F) t-Distributed stochastic neighbor embedding (tSNE) graph of the high-dimensional flow cytometry data, gated on transferred CD8<sup>+</sup> T cells from the spleen and hypothalamus of Orex-HA mice on day 30 after transfer, with the cluster overlay (PhenoGraph) (left) and heatmap of marker expression values (gMFI) in the eight identified clusters. (G) Frequency of the clusters in the spleen and hypothalamus of five Orex-HA mice from one experiment, representative of two. Statistical analyses were performed using multiple Mann-Whitney test with Benjamini-Hochberg correction. \*\**P* < 0.01.

This analysis confirmed the heterogeneity of the hypothalamic HA-specific tissue-resident CD8<sup>+</sup> T cells (Fig. 4F). Unsupervised clustering of HA-specific CD8<sup>+</sup> T cells from spleen and hypothalamus identified eight clusters, of which five (clusters 3, 4, 6, 7, and 8) were specific to the hypothalamus (Fig. 4G). The analysis of the markers associated with these five hypothalamic clusters matched those from the scRNA-seq data. Cluster 6 expressed exhaustion markers including TOX, LAG3, PD-1, and TIM-3, and the proliferation marker KI-67. Cluster 4 was characterized by a high expression of GRANZYME B, LAG3, and PD-1 but absence of other exhaustion markers. Last, clusters 3 and 7 appeared to be related to the IFN signature identified by scRNA-seq, as shown by the high coexpression of BST2 and LY-6A (SCA-1), variable expression of GRANZYME B, and low expression of TOX and PD-1 (Fig. 4, F and G, and fig. S3D).

To understand which cluster could be the main driver of neuronal loss, we measured expression of cytokines and cytotoxic molecules by the three tissue-resident CD8<sup>+</sup> T cell clusters. This analysis revealed that the PD-1-expressing tissue-resident CD8<sup>+</sup> T cell subsets (TOX<sup>+</sup> and TOX<sup>-</sup>) expressed the highest amount of IFN- $\gamma$ , with the PD-1<sup>+</sup> TOX<sup>-</sup> subset showing a greater proportion of both IFN- $\gamma$ /TNF and PERFORIN/GRANZYME B double producers (fig. S3, E and F). These data suggest that the two PD-1-expressing tissue-resident CD8<sup>+</sup> T cell subsets are likely implicated in neuronal loss.

Together, this analysis revealed that autoreactive tissue-resident CD8<sup>+</sup> T cells infiltrating the CNS are phenotypically and functionally heterogeneous.

### Autoreactive brain tissue-resident CD8<sup>+</sup> T cells also develop after vaccine-induced autoimmunity

To assess whether the tissue-resident differentiation of HA-specific CD8<sup>+</sup> T cells within the brain resulted from their in vitro activation conditions, we used a different autoimmune model of neuroinflammation involving in vivo T cell priming (30). Here, Orex-HA mice are cotransferred with naïve HA-specific CD8<sup>+</sup> T cells and naïve HA-specific CD4<sup>+</sup> T cells and are immunized the next day with the Pandemrix influenza vaccine (Fig. 5A). This vaccine was associated with narcolepsy side effect in humans after the 2009–2010 H1N1 influenza vaccination campaign. This vaccine contains the immunogenic K<sup>d</sup>-restricted and I-E<sup>d</sup>-restricted HA peptides recognized by the HA-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively. In this model, vaccination-induced activation of HA-specific T cells resulted in a robust hypothalamic T cell infiltration (Fig. 5B) and progressive loss of orexinergic neurons, reaching 36% by 30 days after vaccination (Fig. 5C), associated with sustained inflammation with presence of MHC-II<sup>+</sup>-activated microglia cells (fig. S4, A and B).

The HA-specific CD8<sup>+</sup> T cells peaked in the hypothalamus of Orex-HA mice 2 weeks after vaccination and persisted for more than 8 weeks, again suggesting a potential T<sub>RM</sub> differentiation (Fig. 5B). The proportion of HA-specific CD8<sup>+</sup> T cells expressing CD69 and CD103 in the hypothalamus increased from 42.4% at 2 weeks after vaccination to 68.8% at 8 weeks (Fig. 5, D to F). Their splenic counterparts did not harbor this phenotype (Fig. 5, D and E), suggesting that the CNS tissue and/or presence of the HA antigen was instrumental for T<sub>RM</sub>-like differentiation. HA-specific CD8<sup>+</sup> T cells selectively exhibited the T<sub>RM</sub> features, whereas the endogenous CNS-infiltrating CD8<sup>+</sup> T cells mostly did not (fig. S4C).

We interrogated our recent transcriptome analysis (bulk RNA-seq) performed at day 14 after vaccination (30) and confirmed that

HA-specific CD8<sup>+</sup> T cells infiltrating the hypothalamus adopted a T<sub>RM</sub> transcriptional program. The set of genes differentially expressed between hypothalamic compared with splenic HA-specific CD8<sup>+</sup> T cells was highly enriched for brain T<sub>RM</sub> gene expression signature (Fig. 5G).

Moreover, gene expression analysis suggested that in vivo priming of HA-specific CD8<sup>+</sup> T cells led to a similar functional outcome as in the adoptive transfer model. We observed an up-regulation of genes encoding T<sub>RM</sub> canonical markers (*Itgae*, *CD69*, *P2xr7*, and *Bhlhe40*), exhaustion markers (*Pdcd1*, *Lag3*, and *Tox*), effector cytokines (*Ifng*, *Tnf*, *Ccl4*, and *Xcl1*), cytotoxic molecules (*Gzmb* and *Gzmc*), and IFN signaling-associated genes (*Ly6a*, *Bst2*, *Stat1*, and *Irf7*) in the hypothalamus-derived cells, and a reciprocal down-regulation of genes involved in T cell tissue egress (*Klf2* and *S1pr1*) and central memory differentiation (*Tcf7*, *Id3*, and *Sell*) (Fig. 5H). Akin to the adoptive transfer model, hypothalamic HA-specific CD8<sup>+</sup> T cells primed by Pandemrix vaccination were more cytotoxic and proliferative as shown by the increased expression of GRANZYME B and KI-67, respectively (fig. S4, D and E).

These data indicate that autoreactive tissue-resident CD8<sup>+</sup> T cells with effector potential can form early on in the CNS in the setting of a vaccine-induced neuroinflammatory disease and are associated with sustained inflammation and loss of orexinergic neurons.

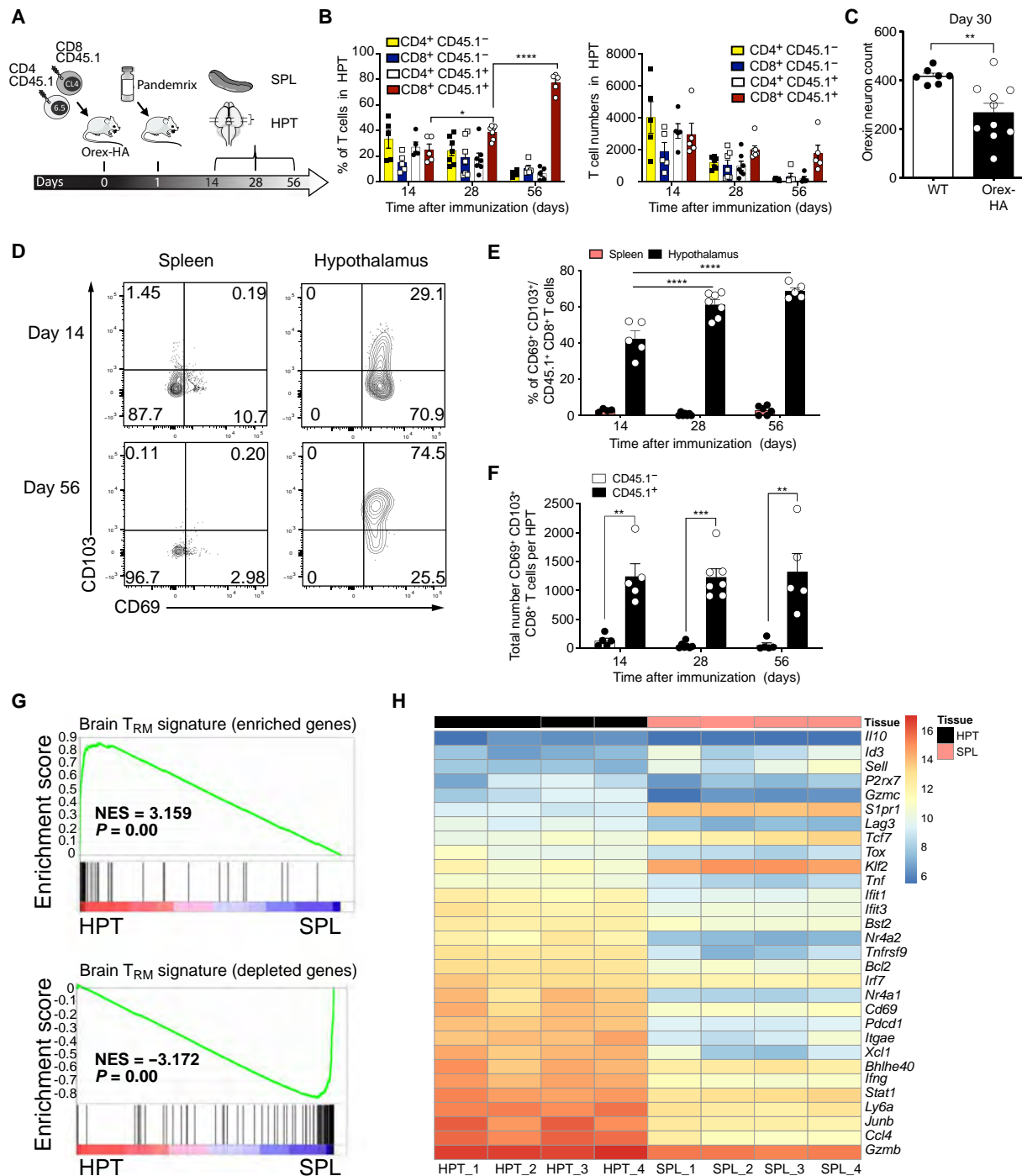
### Recirculating HA-specific effector CD8<sup>+</sup> T cells are dispensable for orexin neuron loss

To investigate the relative contribution of the recirculating compared with hypothalamus-resident HA-specific CD8<sup>+</sup> T cells in the loss of orexinergic neurons, we depleted circulating CD8<sup>+</sup> T cells using an anti-CD8 $\alpha$  depleting antibody starting 8 days after adoptive transfer of activated HA-specific CD8<sup>+</sup> T cells (Fig. 6A). This treatment resulted in a sustained and almost complete elimination of peripheral HA-specific CD8<sup>+</sup> T cells (Fig. 6, B to D, and fig. S5A). However, the proportion and total number of HA-specific tissue-resident CD8<sup>+</sup> T cells in the hypothalamus were not reduced by the peripheral CD8<sup>+</sup> T cell depletion at day 30 (Fig. 6, E to G, and fig. S5B) and up to day 90 (fig. S5C) after transfer, demonstrating that these tissue-resident CD8<sup>+</sup> T cells are long lived, stably maintained within the hypothalamus of Orex-HA mice, and are not dependent on replenishment from peripheral T cells.

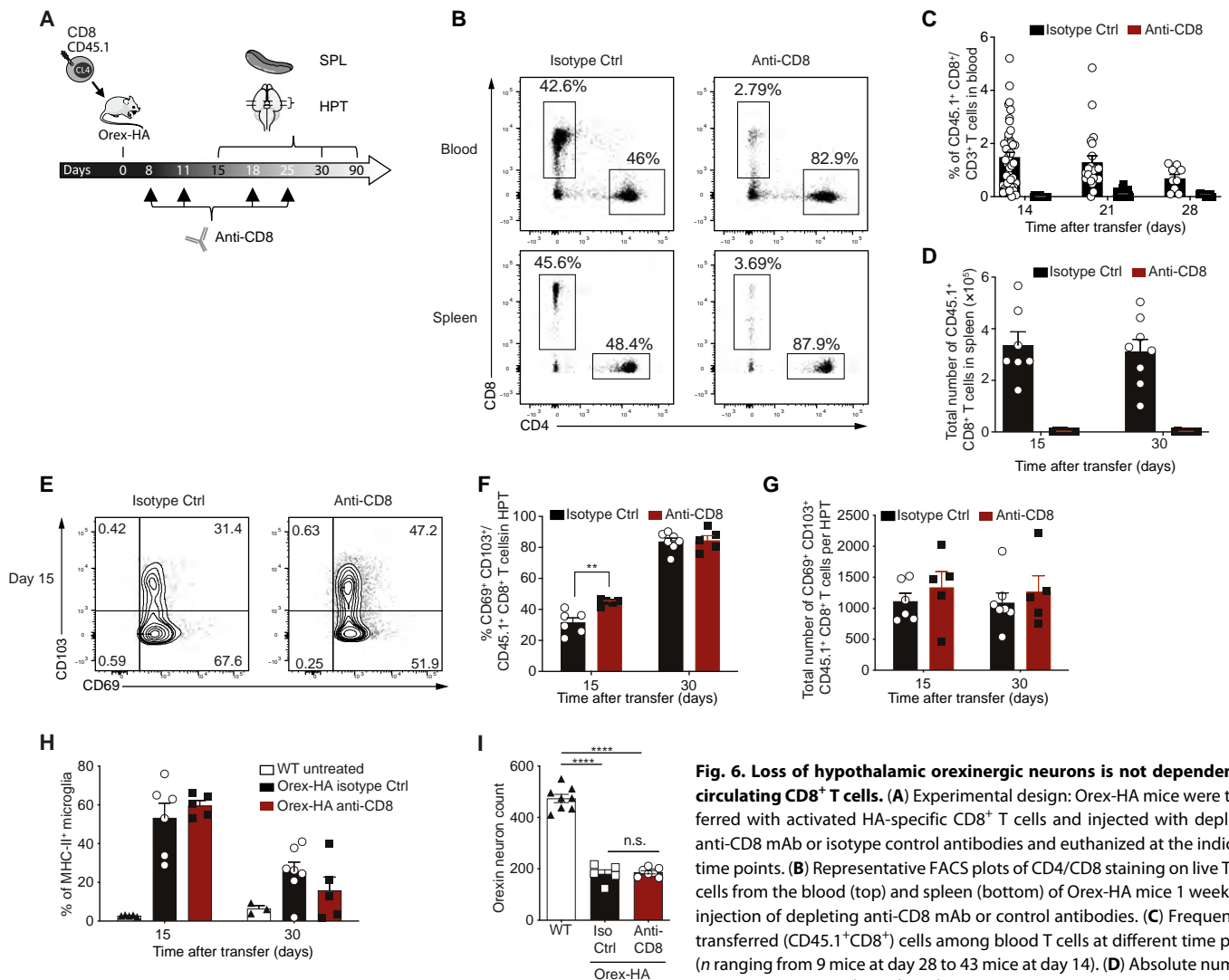
Neither the microglial activation (Fig. 6H) nor the loss of orexinergic neurons (Fig. 6I) was significantly affected ( $P > 0.05$ ) by the depletion of circulating CD8<sup>+</sup> T cells. Together, these data demonstrate that recirculating CD8<sup>+</sup> T cells are not required for the progressive loss of orexinergic neurons and the sustained focal neuroinflammation and suggest that tissue-resident CD8<sup>+</sup> T cells are key for inflammatory tissue damage.

### CD4<sup>+</sup> T cell-mediated maintenance of tissue-resident CD8<sup>+</sup> T cells is required for neuron loss and sustained local inflammation

CD4<sup>+</sup> T cell help is important for the development and/or the maintenance of T<sub>RM</sub> in infection models (31–33). Therefore, we postulated that depletion of CD4<sup>+</sup> T cells in our mouse model would deplete tissue-resident CD8<sup>+</sup> T cells, allowing assessment of their role in autoimmune disease chronicity. CD4<sup>+</sup> T cells were thus depleted starting 5 days before adoptive transfer of HA-specific CD8<sup>+</sup> T cells. To rule out a potential role for peripheral CD8<sup>+</sup> T cells in that setting, we also combined CD4<sup>+</sup> T cell depletion with CD8<sup>+</sup> T cell depletion (starting from day +8, after seeding of the tissue-resident



**Fig. 5. Autoreactive tissue-resident CD8<sup>+</sup> T cells arise in the hypothalamus after in vivo priming.** (A) Experimental design: Orex-HA or WT mice were adoptively transferred with CD45.1<sup>+</sup> naïve HA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells and vaccinated 24 hours later with the Pandemrix flu vaccine. (B) Absolute numbers of T cells and proportion of CD45.1<sup>-</sup> (yellow) and CD45.1<sup>+</sup> (white) CD4<sup>+</sup> T cells, and of CD45.1<sup>-</sup> (blue) and CD45.1<sup>+</sup> (red) CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice, at different time points after vaccination. Graphs show the mean of 5 to 7 data points (12 to 39 mice) per time point from two to three independent experiments. (C) Orexin-expressing hypothalamic neurons were quantified by immunohistochemistry 30 days after vaccination in Orex-HA (*n* = 10) and control (*n* = 7) mice from two experiments. (D) Representative FACS plots and (E) frequency of CD69<sup>-</sup> and CD103<sup>-</sup> expressing splenic and hypothalamic transferred CD8<sup>+</sup> T cells (live CD11b<sup>+</sup>TCRβ<sup>+</sup>CD8<sup>+</sup>CD45.1<sup>+</sup> cells) from Orex-HA mice at different time points after vaccination. (F) Absolute numbers of hypothalamic CD69<sup>+</sup> CD103<sup>+</sup> CD8<sup>+</sup> T cells of either endogenous (CD45.1<sup>-</sup>) or transferred (CD45.1<sup>+</sup>) origin in Orex-HA mice. Each data point (*n* = 5 to 7) is from 2 to 18 pooled hypothalami, and results are from two (day 56) to three (days 14 and 28) independent experiments. (G) GSEA of a published brain TRM signature in a ranked list of genes differentially expressed between hypothalamic versus splenic transferred CD8<sup>+</sup> T cells from Orex-HA mice. (H) Heatmap of the expression of selected genes in transferred CD8<sup>+</sup> T cells from the hypothalamus (black) and spleen (red). Statistical analyses were performed using the Mann-Whitney (C), two-way ANOVA with Tukey's (B) or Sidak's post hoc test (E), and multiple Mann-Whitney test with Benjamini-Hochberg correction (F). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

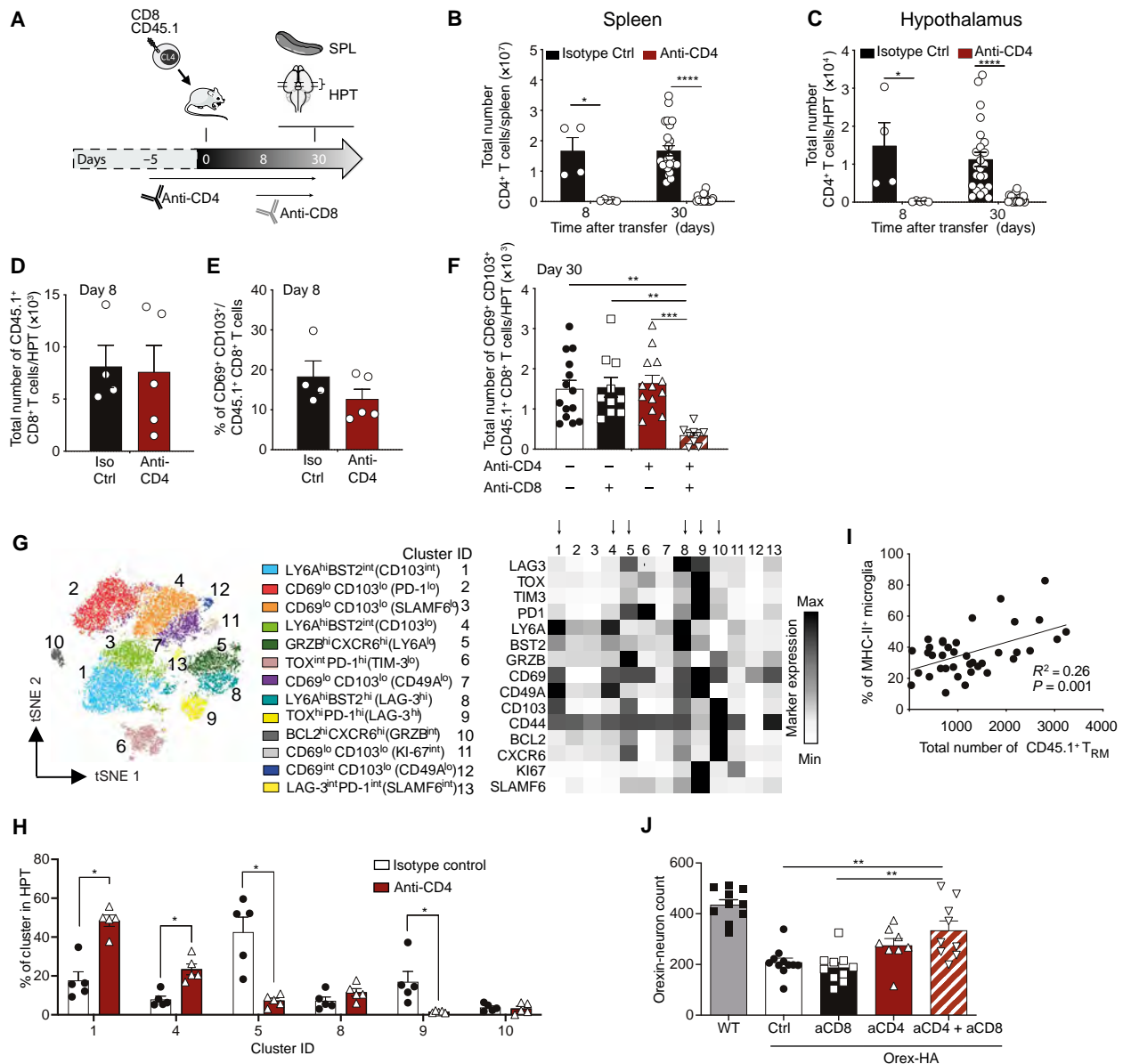


**Fig. 6. Loss of hypothalamic orexinergic neurons is not dependent on circulating CD8<sup>+</sup> T cells.** (A) Experimental design: Orex-HA mice were transferred with activated HA-specific CD8<sup>+</sup> T cells and injected with depleting anti-CD8 mAb or isotype control antibodies and euthanized at the indicated time points. (B) Representative FACS plots of CD4/CD8 staining on live TCRβ<sup>+</sup> cells from the blood (top) and spleen (bottom) of Orex-HA mice 1 week after injection of depleting anti-CD8 mAb or control antibodies. (C) Frequency of transferred (CD45.1<sup>+</sup>CD8<sup>+</sup>) cells among blood T cells at different time points (*n* ranging from 9 mice at day 28 to 43 mice at day 14). (D) Absolute numbers of transferred CD45.1<sup>+</sup>TCRβ<sup>+</sup>CD8<sup>+</sup> T cells in the spleen of Orex-HA mice treated

with depleting anti-CD8 mAb or control antibodies (*n* = 7 to 8 per group per time point). (E) Representative FACS plots on day 15, (F) frequency, (G) absolute numbers of CD69<sup>+</sup> CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells, and (H) MHC-II expression on microglia (live CD45.2<sup>int</sup>CD11b<sup>int</sup>Ly-6c<sup>-</sup> cells) in the hypothalamus of Orex-HA mice treated with anti-CD8 or control antibodies. Each data point (*n* = 5 to 7) is from two pooled mice. Results are from two independent experiments per time point. (I) Immunohistochemistry quantification of orexin-expressing hypothalamic neurons at day 30 in five to eight mice per group from two independent experiments. Statistical analyses were performed using two-way ANOVA with Sidak's post hoc test (F to H) and one-way ANOVA with Tukey's post hoc test (I). Ctrl, control; n.s., not significant. \*\**P* < 0.01 and \*\*\*\**P* < 0.0001.

CD8<sup>+</sup> T cells within the hypothalamus (Fig. 7A). CD4<sup>+</sup> T cells were absent from the periphery and CNS throughout the experiment after administration of depleting anti-CD4 antibody (Fig. 7, B and C, and fig. S6A). The CD4<sup>+</sup> T cell depletion did not reduce the migration of HA-specific CD8<sup>+</sup> T cells to the hypothalamus at day 8 after transfer (Fig. 7D), and it did not prevent their differentiation into CD69<sup>+</sup> CD103<sup>+</sup> T cells (Fig. 7E). At this early time point, the magnitude of microglial cell activation in the hypothalamus was not altered by CD4<sup>+</sup> T cell depletion (fig. S6B). However, at day 30, the number of tissue-resident CD8<sup>+</sup> T cells was strongly reduced upon combined CD4<sup>+</sup> (from day -5) and CD8<sup>+</sup> (from day +8) T cell depletion compared with the other conditions (Fig. 7F), suggesting that CD4<sup>+</sup> T cell depletion affected the persistence of tissue-resident CD8<sup>+</sup> T cells in the CNS but not their initial differentiation.

To further explore the contribution of CD4<sup>+</sup> T cell help on the phenotype and functions of tissue-resident CD8<sup>+</sup> T cells, we used high-dimensional flow cytometric analysis and determined whether the distribution of tissue-resident HA-specific CD8<sup>+</sup> T cells within the previously identified hypothalamic clusters could be affected by the depletion of CD4<sup>+</sup> T cells alone (Fig. 7, G and H) or combined with the depletion of CD8<sup>+</sup> T cells in the periphery (fig. S6C). Cluster 5 (the more cytotoxic PD-1<sup>int</sup> GRANZYME B<sup>high</sup> subset) and cluster 9 (the exhausted TOX<sup>+</sup> PD-1<sup>+</sup> TIM3<sup>+</sup> KI-67<sup>+</sup> cells) were selectively reduced by CD4<sup>+</sup> T cell depletion combined or not with CD8<sup>+</sup> T cell depletion (Fig. 7, G and H, and fig. S6, C and D). By contrast, the proportion of cells in clusters 1 and 4, comprising poorly cytotoxic tissue-resident CD8<sup>+</sup> T cells expressing LY-6A and BST-2 (mostly PD-1<sup>-</sup> TOX<sup>-</sup>), was increased upon CD4<sup>+</sup> T cell depletion (Fig. 7G and fig. S6, C and D).



**Fig. 7. Tissue-resident CD8<sup>+</sup> T cell persistence and their functional properties in the hypothalamus are dependent on CD4<sup>+</sup> T cells and are required for sustained neuronal loss.** (A) Experimental design: Orex-HA mice were treated with depleting anti-CD4 mAb (or control antibodies) starting 5 days before HA-specific activated CD8<sup>+</sup> T cell transfer. From day 8 onward, groups of mice also received depleting anti-CD8 mAb (or control antibodies) until euthanasia. (B) Absolute numbers of CD4<sup>+</sup> T cells in the spleen and (C) hypothalamus in Orex-HA mice treated with anti-CD4 mAb or control immunoglobulin G2b (IgG2b) at different time points after CD8<sup>+</sup> T cell transfer. The data points are from two (day 8; n = 4 to 5 mice per group) or three (day 30; n = 19 to 24 mice per group) independent experiments. (D) Number of transferred CD8<sup>+</sup> T cells and (E) frequency of CD69<sup>+</sup>CD103<sup>+</sup> among them in the hypothalamus of Orex-HA mice treated with anti-CD4 or isotype control antibodies, on day 8, in four to five mice per group from two independent experiments. (F) Numbers of CD69<sup>+</sup>CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with control antibodies, with anti-CD8 and/or anti-CD4 mAb, on day 30. Data are from 9 to 14 mice per group in three independent experiments. (G) tSNE graph with the cluster overlay (left) of transferred CD8<sup>+</sup> T cells from the hypothalamus of the four treatment groups and from the spleen of the IgG2b group on day 30 after transfer, and heatmap of the expression of key markers (right) in the 13 identified clusters. Arrows indicate the main hypothalamic clusters. (H) Frequency of the main hypothalamic clusters in five mice per group from one experiment, representative of two. (I) Correlation, on day 30, between MHC-II expression on microglia and the number of CD69<sup>+</sup>CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice. The correlation using Pearson's test involves 38 mice from the four treatment groups in three independent experiments. (J) Immunohistochemistry quantification at day 30 of hypothalamic orexin-expressing neurons in 8 to 11 mice per group from three independent experiments. Statistical analyses were performed using multiple Mann-Whitney test with Benjamini-Hochberg correction (B, C, and H), Kruskal-Wallis test with Dunn's post hoc test (F), and one-way ANOVA with Tukey's post hoc test (J). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001.

These data indicate that CD4<sup>+</sup> T cell depletion affected both the maintenance and the function of hypothalamic tissue-resident CD8<sup>+</sup> T cells.

Moreover, the combined depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells affected local inflammation and neuronal loss. The proportion of MHC-II<sup>+</sup> microglial cells strongly correlated with tissue-resident CD8<sup>+</sup> T cell numbers (Fig. 7I). Orexinergic neurons were partly preserved in Orex-HA mice in the CD4<sup>+</sup> and CD8<sup>+</sup> T cell double-depleted group (Fig. 7J). Therefore, the data suggest that the CD4<sup>+</sup> T cell-mediated control of long-term persistence and function of tissue-resident CD8<sup>+</sup> T cells within the CNS is essential to drive the chronicity of the autoimmune process.

Next, the phenotype of the host CD4<sup>+</sup> T cells from dissected hypothalamus and spleen of Orex-HA mice was investigated by high-dimensional flow cytometry, 30 days after CD8<sup>+</sup> T cell transfer. Among the nine different CD4<sup>+</sup> T cell clusters identified, cluster 4 represented the dominant hypothalamic subset (24.9%) (fig. S7A). This cluster expressed markers of both tissue residency (CXCR6<sup>high</sup> CD49A<sup>high</sup> and CD69<sup>+</sup>) and T follicular helper cells (T<sub>FH</sub>) (CXCR5<sup>+</sup>, PD-1<sup>+</sup>, and ICOS<sup>+</sup>) (fig. S7, B to D), resembling the tissue-resident helper cells (T<sub>RH</sub>) (33). To assess whether this cluster could contribute locally to the maintenance of tissue-resident CD8<sup>+</sup> T cells, we enumerated HA-specific tissue-resident CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice depleted of peripheral CD4<sup>+</sup> T cells 5 days before CD8<sup>+</sup> T cell transfer (early depletion) or at day 8 after transfer (late depletion). At day 8, host CD4<sup>+</sup> T cells are already detected in the hypothalamus, and we reasoned that systemic injection of depleting anti-CD4 monoclonal antibody (mAb) should spare tissue-resident CD4<sup>+</sup> T cells while eliminating peripheral/recirculating CD4<sup>+</sup> T cells. In addition, peripheral CD8<sup>+</sup> T cells were depleted from day 8 onward to avoid potential contributions from peripheral CD8<sup>+</sup> T cells (fig. S7E). As expected, CD4<sup>+</sup> T cells were absent from the spleen in both early and late CD4<sup>+</sup> T cell depletion and from the hypothalamus upon early depletion (fig. S7F). However, late CD4<sup>+</sup> depletion spared a population of CD4<sup>+</sup> T cells in the hypothalamus expressing CD69, CD49A, and CXCR6, confirming their tissue-resident phenotype (fig. S7, G to I). In addition, we confirmed that a large majority (on average 81.6%) of these CD4<sup>+</sup> T cells coexpressed T<sub>FH</sub> markers (fig. S7, J and K). In contrast to early CD4 depletion, late depletion did not impair tissue-resident CD8<sup>+</sup> T cell maintenance, suggesting that local help provided by the tissue-resident CD4<sup>+</sup> T cells allows persistence of tissue-resident CD8<sup>+</sup> T cells (fig. S7L). This conclusion is supported by microscopy observations of host CD4<sup>+</sup> T in close vicinity with CD103<sup>+</sup> tissue-resident CD8<sup>+</sup> T cells within the hypothalamus (fig. S7M).

We, then, addressed whether the host CD4<sup>+</sup> T cells could be activated in secondary lymphoid organs by antigen leakage resulting from the destruction of HA-expressing neurons. When transferred into Orex-HA or control mice that had received *in vitro* activated HA-specific CD8<sup>+</sup> T cells 12 days earlier, CellTrace Violet (CTV)-labeled naïve HA-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells proliferated 3 days later in the cervical lymph nodes (LNs) and, to lesser extent, in the spleen of Orex-HA mice (fig. S8, A and B). This antigen leakage allowed activation of naïve HA-specific T cells as indicated by higher expression of activation markers in Orex-HA compared with control mice (fig. S8, B and C).

Together our results suggest that (i) tissue-resident CD4<sup>+</sup> T cells provide local help to tissue-resident CD8<sup>+</sup> T cells, thereby allowing their long-term maintenance, and (ii) some of these CD4<sup>+</sup> T cells

are primed by neuronal antigen leakage induced by the first wave of brain-infiltrating autoreactive CD8<sup>+</sup> T cells.

### CD8<sup>+</sup> T cells within lesions of patients with neuron-targeting autoimmune encephalitis express markers present in mouse tissue-resident CD8<sup>+</sup> T cells

To further link the mouse data with human pathology, we performed additional brain immunohistological staining in patients with autoimmune diseases against intracellular neuronal self-antigens, combining the CD8 and CD103 staining with CD69, GRANZYME B, TOX, BCL2, and PD-1 staining (Fig. 8, A to E). CD69 and BCL2 were highly and preferentially expressed in CD8<sup>+</sup> T cells expressing CD103, further underlining their tissue-resident feature (Fig. 8F). Moreover, human CNS-infiltrating CD103<sup>+</sup> CD8<sup>+</sup> T cells also frequently expressed some key molecules delineating specific tissue-resident CD8<sup>+</sup> T cell subsets in our mouse study, such as GRANZYME B, TOX, and PD-1 (Fig. 8F). It is, therefore, possible that, as in the mouse model, these tissue-residing CD8<sup>+</sup> T cells are the drivers of neuronal destruction in human pathologies.

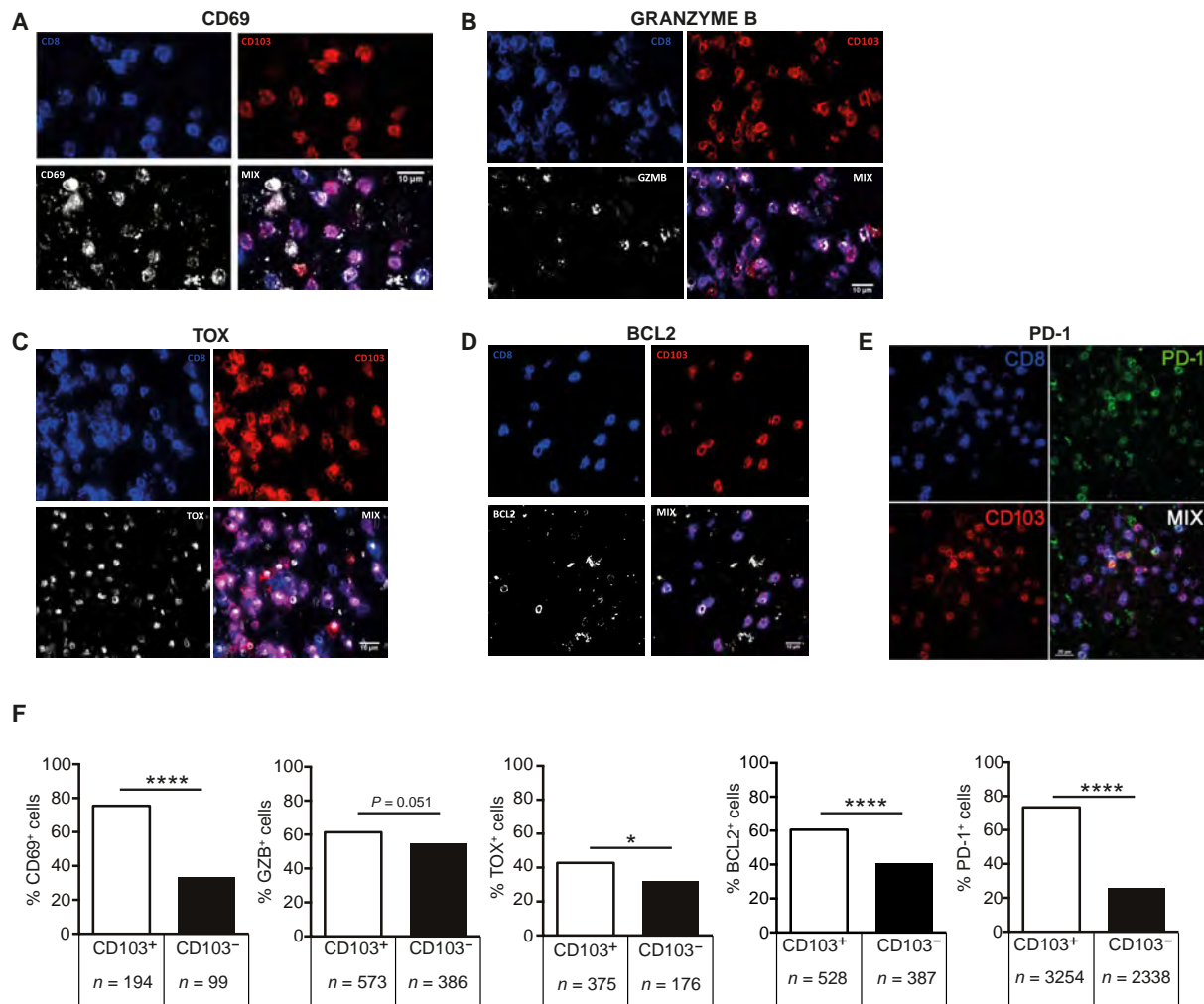
### DISCUSSION

In this study, we analyzed brain tissue from patients with CNS neuron-targeting autoimmune diseases and identified a high proportion of CD8<sup>+</sup> T cells expressing CD103, a prototypic T<sub>RM</sub> marker. We then made use of a mouse model of autoimmunity against CNS neurons to investigate the contribution of tissue-resident CD8<sup>+</sup> T cells in the disease process. We showed that CNS-infiltrating autoreactive effector CD8<sup>+</sup> T cells rapidly acquired a tissue-resident phenotype, remained for months, independently of any supply from the periphery. These cells fueled locally the deleterious autoimmune process, resulting in “brain-autonomous” neuronal degeneration. Tissue-resident CD8<sup>+</sup> T cells critically relied on CD4<sup>+</sup> T cells for their persistence in the CNS, for their cytotoxic properties, and for the progressive autoimmune-mediated neuronal loss. These data suggest that autoreactive tissue-resident CD8<sup>+</sup> T cells have a direct deleterious effect on the self-antigen-expressing neurons and are needed for the chronicity of the disease process.

The concept of the immune privilege of the CNS has been challenged by the identification of several gateways between the peripheral immune system and the brain, despite the restrictive blood-brain barrier (34, 35). In addition, the CNS may enable the differentiation and the survival of tissue-resident immune cells stably lodged *in situ*. This would explain why human chronic inflammatory diseases of the CNS are frequent, ranging from autoimmune to neurodegenerative or infectious. Recent data from scRNA-seq of human cerebrospinal fluid cells from patients with MS and healthy donors indicate that local memory CD8<sup>+</sup> T cells, but also a cluster of memory CD4<sup>+</sup> T cells, express markers of tissue residence and cytotoxicity-related genes (24, 36). These CD8<sup>+</sup> T cells, the clonally expanded ones, share expression of  $\alpha$ E integrin, CD69, CXCR6, and CCR5, suggesting possible therapeutic targeting (24, 36).

T<sub>RM</sub> have been involved in pathogen clearance and/or in the control of pathogen reactivation in infectious diseases of the CNS (31, 32, 37, 38). By contrast, T<sub>RM</sub> phenotype and function have been scarcely documented in the context of CNS autoimmune diseases, and it is unclear whether T<sub>RM</sub> are sustaining the chronicity of neuro-inflammatory diseases. Although T<sub>RM</sub>-like cells have been reported in the active lesions of patients with MS (10, 23) and in Parkinson's





**Fig. 8. Expression of CD69, GRANZYME B, TOX, BCL2, and PD-1 in T<sub>RM</sub>-like CD8<sup>+</sup> T cells infiltrating the parenchyma in human neuron-targeting autoimmune encephalitis.** Microphotographs of CNS-infiltrating CD8<sup>+</sup> T cells either CD103<sup>+</sup> or CD103<sup>-</sup>, illustrating the expression of CD69 (A), GRANZYME B (GZB) (B), TOX (C), BCL-2 (D), and PD-1 (E). Scale bars, 10  $\mu$ m (A to D) and 20  $\mu$ m (E). (F) Histograms showing the percentage of CD103<sup>+</sup> and CD103<sup>-</sup> CD8<sup>+</sup> T cells expressing CD69 (two patients, 293 cells), GRANZYME B (nine patients, 959 cells), TOX (four patients, 551 cells), BCL2 (nine patients, 915 cells), and PD-1 (six patients, 5592 cells). Statistical analyses were performed using chi-square test with Yates' continuity correction. \**P* < 0.05 and \*\*\*\**P* < 0.0001.

disease (39), their contribution to pathology and their antigenic specificity are still elusive. In addition, whether T<sub>RM</sub> could be found in other CD8<sup>+</sup> T cell-mediated autoimmune diseases of the CNS is unknown. To tackle these issues, we analyzed the presence of T<sub>RM</sub>-like cells in brain tissue of patients suffering from severe autoimmune diseases, characterized by CD8<sup>+</sup> T cell-mediated autoimmune targeting of neurons. A high density of T<sub>RM</sub>-like cells was found in these samples, suggesting that T<sub>RM</sub> differentiation is likely a hallmark of these conditions.

We showed, in an experimental model, that antigen-specific tissue-resident CD8<sup>+</sup> T cells formed rapidly after their entry in the CNS and resided in the hypothalamus for up to 3 months. Critically, the presence of tissue-resident CD8<sup>+</sup> T cells was associated with the progressive loss of orexinergic neurons over time, which correlated with microglial activation. Conversely, depletion of peripheral CD8<sup>+</sup> T cells did not prevent loss of the orexinergic neurons, indicating that, once the tissue-resident CD8<sup>+</sup> T cells are established, peripheral CD8<sup>+</sup> T cells are dispensable. Higher numbers of tissue-resident

CD8<sup>+</sup> T cells were present in the hypothalamus than in the cerebellum, a site devoid of orexinergic neurons, indicating that antigen presentation is essential for the compartmentalized accumulation of tissue-resident CD8<sup>+</sup> T cells.

To decipher whether tissue-resident CD8<sup>+</sup> T cells were directly involved in the loss of neurons and in the long-lasting compartmentalized inflammatory process, we depleted CD4<sup>+</sup> T cells before the HA-specific CD8<sup>+</sup> T cell transfer. CD4<sup>+</sup> T cells had been shown to promote T<sub>RM</sub> differentiation and/or survival in infectious settings (31–33). Our data revealed that when CD4<sup>+</sup> T cell depletion was combined with peripheral CD8 T cell depletion, hypothalamic tissue-resident CD8<sup>+</sup> T cell numbers were markedly reduced, and, reciprocally, orexin neurons were partially protected. However, CD4<sup>+</sup> T cell depletion alone did not reduce the number of tissue-resident CD8<sup>+</sup> T cells. A possible explanation is that in the absence of help from CD4<sup>+</sup> T cells, tissue-resident CD8<sup>+</sup> T cells are unable to persist but are progressively replaced by peripheral CD8<sup>+</sup> T cells, refilling the niche left free by “unhelped” CD8<sup>+</sup> T cells. These results are

congruent with experimental brain infection with Poliovirus, in which virus-specific  $T_{RM}$  were unable to persist in the combined absence of peripheral  $CD8^+$  T cells and interleukin-21 (IL-21)-producing  $CD4^+$  T cells expressing  $T_{FH}$  surface markers (31, 32). The  $CD4^+$  T cell subset likely providing help to tissue-resident  $CD8^+$  T cells in the brain expressed  $T_{FH}$  surface markers and tissue-resident phenotype, as described in the lung after influenza virus infection (33). The priming of  $CD4^+$  T cells could be secondary to the neuronal damage caused by the first wave of autoreactive  $CD8^+$  T cells, leading to neuronal antigen leakage in the cervical LNs. This suggests that chronic CNS inflammation could be fueled by a positive feedback loop in which neuronal destruction by  $CD8^+$  T cells leads to de novo priming of  $CD4^+$  T cells promoting, in turn, the long-term persistence and effector functions of tissue-resident  $CD8^+$  T cells.

In the context of autoimmunity, a key question is how to best target the  $T_{RM}$  compartment for therapeutic purposes. As peripheral T cells can replace  $T_{RM}$ , both peripheral and tissue-resident compartments need to be considered. A recent report has detected, among the pool of peripheral memory precursor cells, precursors of  $T_{RM}$ , which exhibit an increased ability to form de novo  $T_{RM}$  (40). One could speculate that both the peripheral  $T_{RM}$  precursors and the mature CNS  $T_{RM}$  should be targeted for durable elimination of pathogenic autoreactive  $T_{RM}$ . Anti-CD20 mAbs, one of the most efficient treatment of MS, deplete not only CD20-expressing B cells but also CD20(dim)-expressing  $CD8^+$  T cells in the peripheral blood (41). CD20<sup>dim</sup> T cells infiltrating MS lesions are strongly enriched in cells expressing  $T_{RM}$  markers ( $CD69^+$   $CXCR6^+$ ) (42). It is therefore possible that the depletion of these CD20-expressing  $T_{RM}$  or their peripheral precursors might contribute to the efficacy of anti-CD20 therapy.

To further characterize the phenotype and function of hypothalamic tissue-resident  $CD8^+$  T cells, we combined single-cell RNA transcriptomic analysis and high-dimensional flow cytometric analysis. These approaches uncovered the heterogeneity of the hypothalamic tissue-resident  $CD8^+$  T cells, encompassing three phenotypically and functionally distinct clusters. The first cluster was characterized by high expression of the cytotoxic molecule GRANZYME B. Conversely, cells from the second cluster were poorly cytotoxic and expressed multiple exhaustion-associated molecules (TOX, PD-1, and TIM-3), the proliferation marker KI-67, and the effector molecule IFN- $\gamma$ . Last, we uncovered a cluster of tissue-resident  $CD8^+$  T cells characterized by high expression of many type I IFN-responsive genes. We observed that the proportion of both the “GRANZYME B<sup>+</sup>” and “TOX<sup>+</sup>” clusters were substantially reduced upon early  $CD4^+$  T cell depletion, whereas the “IFN I signaling” cluster reciprocally increased. Although TOX expression can control T cell exhaustion during chronic lymphocytic choriomeningitis virus (LCMV) infection (43), it is also induced after T cell receptor (TCR) activation similar to PD-1 and TIM3 and has recently been described as a determinant of the encephalitogenic potential and persistence of self-reactive  $CD8^+$  T cells in experimental CNS autoimmunity (43).

With the recent advance in single-cell transcriptome profiling, it has become clearer that  $T_{RM}$  form a heterogeneous T cell pool and that the residency program does not prevent T cells from acquiring specialization programs (44). The mechanisms underpinning  $T_{RM}$  heterogeneity are still poorly understood, although the type of antigen-presenting cells (APCs) restimulating  $T_{RM}$  within the tissue has been shown to influence their transcriptional programming.

Antigen presentation by nonhematopoietic cells to  $T_{RM}$  in the lungs during an influenza virus infection was associated with repression of type I IFN-inducible genes and, conversely, an increased expression of *Ifny*, *Xcl1*, or *Ccl4* (29), all highly expressed by the TOX<sup>+</sup> tissue-resident  $CD8^+$  T cell cluster in our model. We propose that heterogeneity observed among hypothalamic tissue-resident  $CD8^+$  T cells could reflect direct antigen presentation by neurons compared with cross-presentation by professional APCs migrating to the brain.

Our study, together with the companion article (45), demonstrates the important contribution of tissue-resident  $CD8^+$  T cells to the chronicity of destructive CNS autoimmune experimental disease. Moreover,  $CD8^+$  T cells harboring a  $T_{RM}$ -like phenotype and expressing key functional molecules uncovered in the mouse models were prevalent within brain tissues of patients suffering from neuron-targeting autoimmune diseases.

Limitations from our study include the fact that the autoreactive potential of the human  $T_{RM}$ -like cells was not investigated, although their high expression of GRANZYME B and PD-1 suggests recent antigenic stimulation rather than bystander activation. Moreover, we have not explored, in the mouse model, whether the priming phase of autoreactive  $CD8^+$  T cells can affect their differentiation into  $T_{RM}$ . In particular, we did not assess whether an infection with the influenza virus would result in a similar phenotype as in vivo activation of HA-specific T cells with the adjuvanted influenza vaccine. Last, another limitation of our study is that we have not yet addressed to what extent brain-resident  $CD4^+$  T cells induce the loss of neurons, independently of their role in promoting  $CD8^+$   $T_{RM}$  maintenance and effector functions. Further investigations will be needed to decipher the relative contribution and the interplay of  $CD4^+$   $T_{RM}$  and  $CD8^+$   $T_{RM}$  in the context of chronic CNS inflammation. A better understanding of the mechanisms underpinning the adaptation and survival of  $T_{RM}$  within the CNS and of their functional heterogeneity represents therefore important opportunities for future therapies against T cell-mediated neuroinflammatory diseases.

## MATERIALS AND METHODS

### Study design

Our research objective was to assess the contribution of  $CD8^+$   $T_{RM}$  in autoimmune destruction of CNS neurons. We studied postmortem samples from 10 patients suffering from autoimmune diseases directed against neuronal antigens (ethical committee numbers: EK1206/2013-EK1123/2015 and AC-2019-3465-AC-2020-3918). We used transgenic mice expressing a neuron-specific neo self-antigen as well as in vitro- or in vivo-generated effector  $CD8^+$  T cells targeting the model autoantigen. We characterized the CNS-infiltrating autoreactive  $CD8^+$  T cells using single-cell transcriptomics, flow cytometry, and histology and uncovered their tissue-resident nature and their heterogeneity. We used antibody-mediated depletion to evaluate the dependency of autoreactive tissue-resident  $CD8^+$  T cells on  $CD4^+$  T cells and circulating  $CD8^+$  T cells. The time points were prospectively selected. Although no formal power analysis was performed, the number of data points, the number of experimental replicates, and the statistical methods are indicated in the figure legends. Animals were randomly assigned to the different treatment groups. A single animal was excluded because of a likely genotyping error (Fig. 7, F to I). Counting of surviving neurons was performed either in an automated manner or by an investigator blinded to the treatment group. For further details, see the Supplementary Materials.

## Mice

The (BALB/cJ × C57Bl/6J)F1 Orex-HA mice express HA of an H1N1 influenza virus in orexinergic neurons (26). The TCR transgenic mice (BALB/cJ × C57Bl/6J)F1 CL4-TCR and 6.5-TCR, expressing K<sup>d</sup>-restricted HA<sub>512–520</sub>-specific and I-E<sup>d</sup>-restricted HA<sub>110–119</sub>-specific TCR, respectively, have been previously described (30). The TCR transgenic mice express the CD45.1 congenic marker, whereas the recipient mice are CD45.2. Male and female mice (5.5 to 8 weeks old) were included in the experiments. All animal experiments were performed in the UM06S animal facility, Toulouse, France, in accordance with the European Union guidelines after the approval of the local ethics committee (17-U1043 RL/HN-939).

## In vitro T cell differentiation and adoptive transfer of activated CD8<sup>+</sup> T cells

HA-specific CD8<sup>+</sup> T cells were isolated from spleen and LNs of CL4-TCR mice by negative selection using the Dynabeads Untouched Mouse CD8 Cells kit (Invitrogen). Purified CD8<sup>+</sup> T cells were cultivated with irradiated syngeneic splenocytes at a 1:5 ratio in Dulbecco's modified Eagle's medium GlutaMAX medium supplemented with 10% fetal calf serum in the presence of HA<sub>512–520</sub> peptide (1 µg/ml), IL-2 (1 ng/ml), and IL-12 (20 ng/ml) (all from R&D Systems). After 5 days of culture, living cells were collected by Ficoll density separation, and 3 × 10<sup>6</sup> activated CD8<sup>+</sup> T cells were adoptively transferred intravenously in Orex-HA or HA-negative littermate control mice. The activated CD8<sup>+</sup> T cell suspensions routinely contained >95% of CD8<sup>+</sup>Vβ8<sup>+</sup>, and >70% were IFN-γ/TNF producers.

## mAb-depleting treatments

To deplete CD8<sup>+</sup> T cells, an anti-CD8α mAb (clone YTS169.4; In Vivo BioXcell) was injected intraperitoneally starting 8 days after transfer of activated CD8<sup>+</sup> T cells (200 µg per mouse), then at day 11, and weekly (100 µg per mouse) until euthanasia. To deplete CD4<sup>+</sup> T cells, an anti-CD4 mAb (clone GK1.5; In Vivo BioXcell) was injected intraperitoneally 4 days (200 µg per mouse) and 1 day (100 µg per mouse) before or 8 days after adoptive transfer of activated CD8<sup>+</sup> T cells, and then weekly (100 µg per mouse) until euthanasia. For depletion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the two above-described protocols were combined.

## Isolation and stimulation of hypothalamus-infiltrating mononuclear cells

Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused intracardially. Brains were removed, and a hypothalamic block was dissected out, homogenized, and digested in Hanks' balanced salt solution medium with collagenase D (1 mg/ml), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK) (30 µg/ml), and deoxyribonuclease (DNase) I (20 µg/ml), all from Roche Diagnostics. After digestion, the hypothalamic cells were suspended in 30% Percoll (GE Healthcare) and spun at a centrifugation step of 1590g for 30 min without brake. Myelin and debris were removed, and cell suspensions were further used for experiments.

## Histopathology

Immunofluorescence assays on human and mouse brains were performed on 5-µm-thick sections. Antibody concentrations and pretreatments are listed in table S3, and information for staining and cell quantification is provided in the Supplementary Materials.

## RNA extraction and high-throughput scRNA-seq

Thirty days after transfer of activated HA-specific CD8<sup>+</sup> T cells, the HA-specific CD8<sup>+</sup> T cells (CD11b<sup>−</sup>NK1.1<sup>−</sup>CD45.2<sup>+</sup>CD8<sup>+</sup>TCRβ<sup>+</sup>CD45.1<sup>+</sup>) were isolated by fluorescence-activated cell sorting (FACS) (BD FACSAria Fusion) from the spleen and hypothalamus of 13 Orex-HA mice. Immediately after cell sorting, cells were subjected to scRNA-seq processing (10X Genomics, Chromium Single Cell 3' v3 kit) according to the manufacturer's instructions. Sequencing was performed on one flow cell of Illumina NovaSeq 6000 sequencer on 3922 splenic and 1421 hypothalamic CD45.1<sup>+</sup>CD8<sup>+</sup> T cells.

## Statistical analyses

Quantitative data are presented as means ± SEM or percentages. Statistical analyses were performed using GraphPad Prism 9. Student's *t* test, chi-square test, and one-way or two-way analysis of variance (ANOVA) were performed with the appropriate post hoc tests. For nonnormally distributed data (Shapiro-Wilk test, *P* < 0.05), the non-parametric Mann-Whitney and Kruskal-Wallis tests were used. Only two-sided *P* values ≤ 0.05 were considered statistically significant and are indicated on the figures as follows: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

## SUPPLEMENTARY MATERIALS

[www.science.org/doi/10.1126/scitranslmed.abl6157](http://www.science.org/doi/10.1126/scitranslmed.abl6157)

Materials and Methods

Figs. S1 to S8

Tables S1 to S3

Data file S1

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D.F., L.K., A.Y., A.P., K.M.M., D.M., R.H., V.D., J.B., F.M., and R.S.L. analyzed the data and provided critical insight or reagents. F.M. and R.S.L. wrote the first draft of the paper. D.F., A.P., L.K., C.G.-F., D.M., and J.B. edited the paper. All authors reviewed and approved the manuscript.

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associated with this study are present in the paper or the Supplementary Materials. The RNA-seq data have been deposited in GEO (accession number GSE195600) and ArrayExpress (accession number E-MTAB-11416).

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## Tissue-resident CD8<sup>+</sup> T cells drive compartmentalized and chronic autoimmune damage against CNS neurons

David FrieserAurora PignataLeila KhajaviDanielle ShlesingerCarmen Gonzalez-FierroXuan-Hung NguyenAlexander YermanosDoron MerklerRomana HöftbergerVirginie DesestretKatharina M. MairJan BauerFrederick MassonRoland S. Liblau

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### A local contribution to CNS autoimmunity

Aberrantly activated tissue-resident memory T cells (T<sub>RM</sub>) have been shown to contribute to inflammatory conditions.

Their role in the CNS remains unclear. Now, in two complementary studies, Vincenti *et al.* and Frieser *et al.* investigated the role of T<sub>RM</sub> in the CNS. Vincenti and colleagues reported that after viral brain infection, T<sub>RM</sub> triggered CNS inflammation, promoting autoimmune reactions in mice. Cells with T<sub>RM</sub>-like phenotype were also identified in brain tissue from patients with CNS autoimmune diseases. Frieser *et al.* used rodent models of CNS autoimmunity to show that pathogenic CD8<sup>+</sup> T cells infiltrating the CNS adopted a T<sub>RM</sub> phenotype that contribute to the disease. The results suggest that targeting T<sub>RM</sub> can be effective in treating CNS autoimmune diseases.

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Supplementary Materials for  
**Tissue-resident CD8<sup>+</sup> T cells drive compartmentalized and chronic  
autoimmune damage against CNS neurons**

David Frieser *et al.*

Corresponding author: Frederick Masson, frederick.masson@inserm.fr; Roland S. Liblau, roland.liblau@inserm.fr

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**The PDF file includes:**

Materials and Methods  
Figs. S1 to S8  
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References (46–50)

**Other Supplementary Material for this manuscript includes the following:**

Data file S1

## **Supplementary Materials and Methods.**

### **Adoptive transfer of naïve HA-specific T cells and vaccination**

Naïve HA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified from 6.5-TCR and CL4-TCR F1 mice, respectively. Spleens and lymph nodes were collected and T cells were purified (Dynabeads Untouched Mouse CD4 or CD8 Cells kit; Invitrogen). CD4<sup>+</sup> T cells were also incubated with anti-CD25 mAb (clone PC61) for Treg depletion. Naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells were further purified based on expression of CD62L (Miltenyi Biotech). Then, 5x10<sup>6</sup> cells from each population were co-injected i.v. into recipient mice.

Twenty-four hours after adoptive transfer of HA-specific T cells, Orex-HA and littermate control recipient mice were immunized intramuscularly, in the gluteal muscle, with 50 µL of Pandemrix vaccine containing inactivated H1N1 influenza A/California/07/2009 split virion (kindly provided by GlaxoSmith Kline) as described (30).

### **Flow cytometry clustering**

Preprocessed data were down-sampled to a maximum of the sample with the lowest cell number. CD8, CD45.1, CD4, FoxP3, TCRβ and Viability Dye were removed from the t-SNE and Phenograph analysis. Two-dimensional tSNE projections run with the following parameters: perplexity = 30 (the number of nearest neighbors), iteration = 1000 (maximum number of iterations) and *Eta* = 44,099. All clustering were performed with the Phenograph algorithm on the combined dataset according to k-nearest neighbors to enable identification of small populations (46).

### **Immunohistochemistry of mouse brain slices, imaging and data analysis**

For the CD8, CD103 and Orexin triple labeling, brain slices were incubated at 4 °C overnight with primary mouse-anti-Orexin antibodies. Subsequently, the biotinylated secondary system and the tyramide-based amplification of the biotin signal were used. As two out of three antibodies belong to the same species (anti-Orexin and anti-CD8α), the slides were



steamed in EDTA (0.05 M) in Tris buffer (0.01, pH 8.5) for 60 min in order to deactivate the first primary antibody. The sections were then incubated with Cy5-conjugated-avidin for 60 minutes. This was followed by a second overnight incubation with both rabbit anti-CD8 $\alpha$  and goat anti-CD103. Eventually, the sections were incubated for 60 min with Cy2- and Cy3-conjugated secondary antibodies.

For neuronal enumeration, brains were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 30- $\mu$ m-thick coronal free-floating sections were made using a microtome (Leica RM2145). Sections were then incubated with Xylene and EtOH solutions for 5min and 1min respectively and incubated at 118°C in Tris-pH9 solution during 20min for heat-induced epitope retrieval. After incubation with Peroxidase blocking solution (DAKO) for 10min followed by incubation with Block-Buster solution (Innovex) for 20min, slices were incubated overnight at 4°C with 1/2000 anti-Orexin-A primary antibody solution (Phoenix H-003-30). Slices were then incubated with 1/200 secondary antibody solution (E0431 DAKO) for 30min at RT and then incubated with HRP solution (Vector SP-7100) and DAB solution (Kit SK-4104) for 30min and 5min respectively. Orexin<sup>+</sup> neuron counts was performed on 1/10<sup>th</sup> of the entire hypothalamus (10 sections per hypothalamus) using the CaseViewer software (3DHistec Ltd).

### **Immunohistochemistry of human brain slices, imaging and data analysis**

Labeling was performed using the Akyoa Fluorescent Multiplex immunohistochemistry system. After deparaffination with xylene and rehydration, the sections were fixed with 4% Paraformaldehyde (PFA) for 20 minutes and rinsed with distilled water. Before applying the primary antibody, antigen retrieval was performed by heating the sections in AR9 for 60 minutes in a household food steamer (Braun) followed by a 10-minute blocking step with Opal Antibody Diluent/Block solution (AKOYA Bioscience). The primary antibodies were applied either for 2 hours at room temperature or overnight at 4°C. After each incubation

step with the primary antibodies, the sections were rinsed in TBST and subsequently incubated with Opal Polymer HRP Ms + Rb (PerkinElmer) for 10 minutes at room temperature followed by incubating the sections with the different Opal fluorophores for 10 minutes at room temperature. Afterwards, the sections were fixed with 4% PFA followed by another antigen retrieval step using AR6 for 30 minutes before applying the next antibody. Sections were examined using a confocal laser microscope (Leica SP5, Leica) and scanned using the Vectra Polaris Automated Quantitative Pathology Imaging system from Perkin Elmer.

For the quantification of the density of T cells, the proportion of CD8<sup>+</sup> cells among T cells and the frequency of CD103-expressing CD8<sup>+</sup> T cells in patients with autoimmune diseases targeting intracellular neuronal self-antigens, 5 to 26 regions were selected per patient. In each section the CD3<sup>+</sup>, the CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD103<sup>+</sup> cells were counted manually. The total number of cells was divided by the cumulative surface of the different regions.

For the other analyses, two different methods were used for cell quantification, one for PD-1 marker and the other one for GRANZYME B, TOX, BCL2 and CD69 markers. The quantification of the staining was performed with QuPath v0.3.0 software according to the user manual for multiplex analysis.

For the analysis related to CD8, CD103 and PD1 labeling, cells were detected by using the DAPI channel and a channel was created for each marker. Qupath provides a machine-learning tool, which was used to create a classifier for each marker. Prior to saving this algorithm, the selection of cells was inspected manually to verify the accuracy of the classifier. Once the classifier was trained for each marker, a region of interest of the various human encephalitis samples was selected and the algorithm was applied. As Qupath allows to apply several classifiers at the same time, the following combinations of markers were

analyzed:  $CD8^+CD103^+$ ,  $CD8^+CD103^+PD1^+$  and  $CD8^+CD103^+PD1^-$  as well as  $CD8^+CD103^-$ ,  $CD8^+CD103^-PD1^+$  and  $CD8^+CD103^-PD1^-$ . Finally, the absolute numbers of quantified cells were divided by the surface area analyzed.

For the quantification of immunohistochemistry data related to GRANZYME B, TOX, BCL2, and CD69 in human cases, a set of 2-3 random brain regions, containing  $CD8^+$  T cell infiltration, was analyzed for each human patient. To perform this analysis, the perimeter of the analyzed cells was defined using CD8 staining and the mean intensity of CD103, GRANZYME B, TOX, BCL2 and CD69 was measured inside the annotated cells. The distinction between cells positives or negatives for a given marker was defined calculating a cut off. This cut off ( $C$ ) is defined by

$$C = \bar{m}_{neg} + \Delta_{neg}$$

where  $\bar{m}_{neg}$  and  $\Delta_{neg}$  are respectively the average intensity and the standard deviation of a random group of 10 to 15 negative cells of a given image. We also used a simple way of reducing the effects of false negative or false positive cells in the final count. Indeed, when the intensity of the cell ( $I_{cell}$ ) is close to the cutoff, specifically in a  $\frac{\Delta_{neg}}{4}$  range, only  $\frac{4|I_{cell}-C|}{\Delta_{neg}}$  is added to the positive or negative cell group sum (note that this value is inferior to 1 and is equal to 0 when  $I_{cell} = C$ ).  $CD8^+$  T cells were first distinguished between  $CD103^+$  and  $CD103^-$ . Then, the expression of GRANZYME B, TOX, BCL2 and CD69 was independently evaluated within the two groups. Only tissues with reliable staining were included in the quantification data.

### **Single-cell RNA sequencing data analysis.**

FastQ reads were aligned to the reference transcriptome (mm10-3.0.0) with Cell Ranger (10x genomics). Directories containing the output filtered featured matrices were supplied to the functions `Read10x` and `CreateSeuratObject` from the R package `Seurat` (v4.0.1), which are

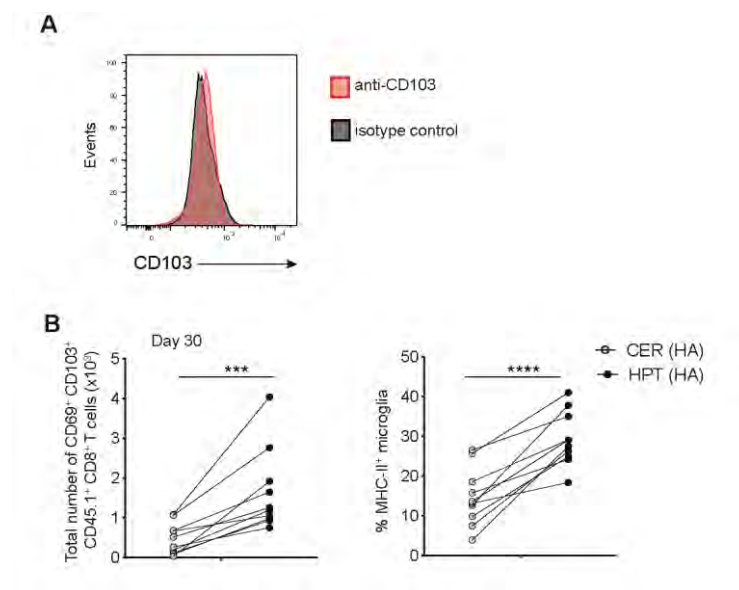
nested in the GEX\_automate function as previously described (47). Briefly, cells containing more than 20% mitochondrial counts were filtered before the data was log normalized by a scaling factor of 10000 and subsequently supplied to the ScaleData function under default parameters. Two-thousand variable features were selected using the 'vst' selection method used for principal component analysis (PCA). The first ten dimensions supplied to Seurat's FindNeighbors function and clustering was performed using a resolution of 0.5. Uniform manifold approximation projection (UMAP) was calculated using the first ten dimensions. Differential gene expression analysis between groups and between clusters was performed using the Wilcoxon Rank Sum test in the FindMarkers function with the logfc.threshold set to 0.25. Cluster defining genes were restricted to the output of the FindMarkers function and were subsequently ranked according to average log fold-change. Heatmaps, feature plots, and violin plots were produced by supplying genes of interest to the functions DoHeatmap, FeaturePlot, and VlnPlot functions in Seurat, respectively. Both cluster defining genes and differentially expressed genes were used to perform gene set enrichment analysis using the GEX\_GSEA function in the R package Platypus (v.3.1) (47), which relies on the fgsea R package (48) and uses the adaptive multilevel splitting Monte Carlo approach. For gene set enrichment analysis, selected gene sets (27, 49, 50) were ordered by p-value and the 300 most significant genes were selected from both the negative and positive log fold-change conditions. GSEA-associated feature and violin plots were created using the top 12 genes based on the leading edge of the enrichment. Volcano plots were produced by supplying the FindMarkers function output with logfc.threshold set to 0 to the GEX\_volcano function from Platypus (47), where colored genes correspond to an adjusted p value < 0.01 and an average log fold change > 0.25. Gene set enrichment and pathway analysis were performed by supplying the top differentially expressed genes by log fold change to the goana and gokegg functions in the R package edgeR.

## **RNA extraction, high-throughput sequencing and data analysis of the RNA sequencing of the vaccine-activated HA-specific CD8<sup>+</sup> T cells**

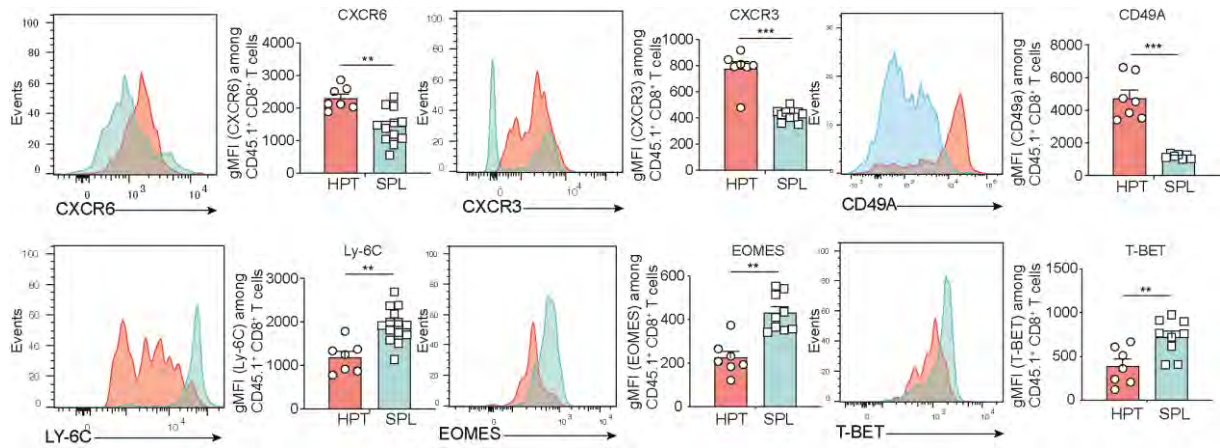
Fourteen days after Pandemrix vaccination of Orex-HA and control recipient mice, transferred (CD45.1<sup>+</sup>) CD44<sup>high</sup>CD62L<sup>low</sup> CD8<sup>+</sup> T cells were isolated from spleen and hypothalami by FACS sorting in 4 independent experiments as described (30). Total RNA was extracted from the sorted cells using RNeasy Micro Kit (Quiagen) and library. Preparation and RNA-sequencing were performed by the Genomics Facility Basel in Zurich, as described (30).

Sequences were quality checked using FastQC (version V0.11.2) and FastqScreen (version V0.11.4) prior to aligning to the *Mus musculus* primary genome sequence (Gencode: GRCm38.p5, release M16) using STAR (version V2.4.0i). FastQC, featureCounts (V1.4.5-p1) and PicardTools (version V2.17.3) were used to assess the mapping quality. RSEM (V1.3.0) was used to generate the expression matrix, which was then analyzed with DESeq2 (R version V3.4.4, BioConductor version V3.6) to identify the differentially expressed genes (DEGs) between hypothalamic and splenic transferred CD8<sup>+</sup> T cells from Orex-HA mice. The GSEA against a published brain TRM signature (27) was performed using ClusterProfiler (R package). A gene list relevant for the tissue-resident signature was extracted.

## Supplementary Figures:

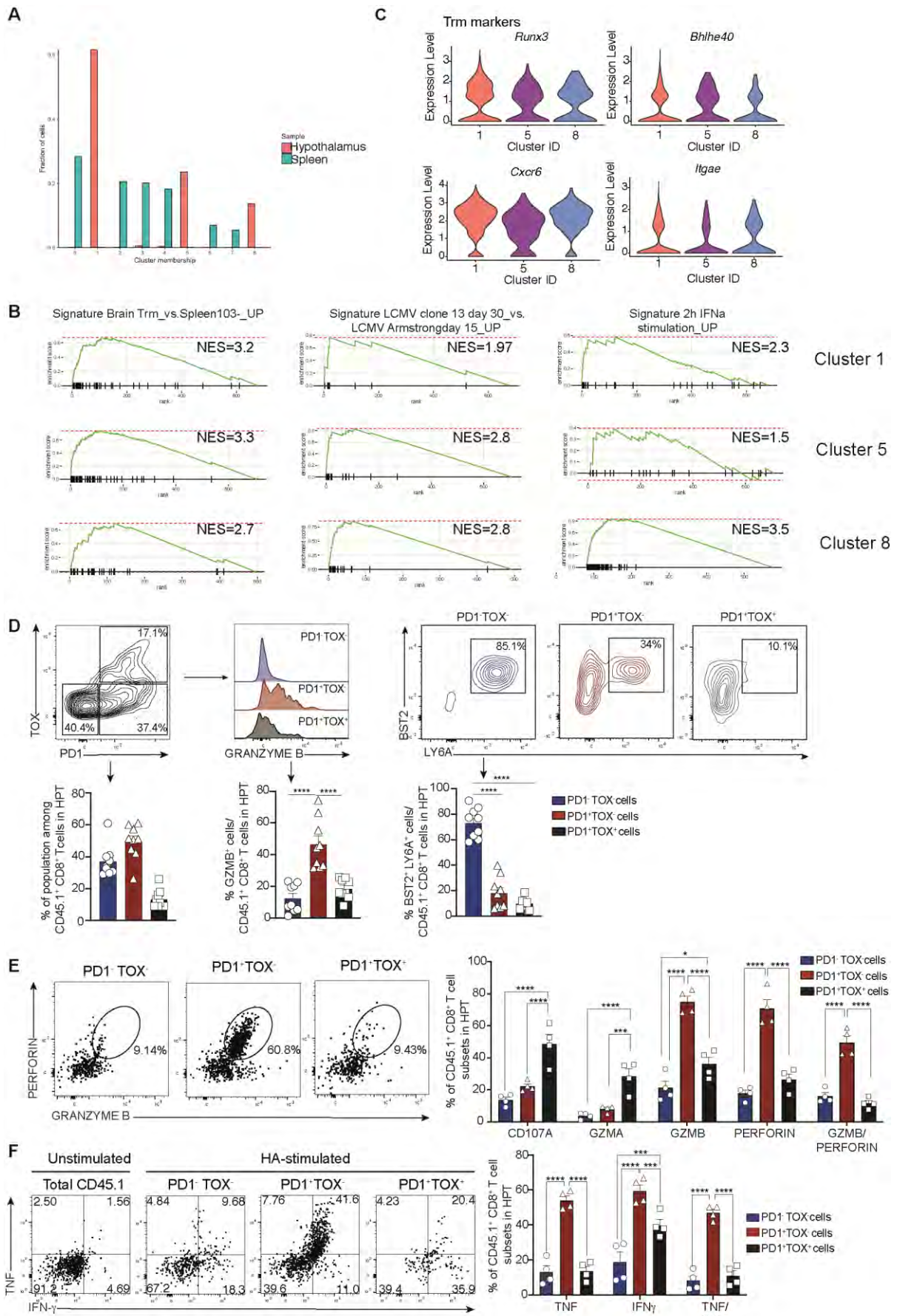


**Figure S1. Transferred CD8<sup>+</sup> T cells with a tissue-resident phenotype are preferentially located in the hypothalamus of Orex-HA mice. (A)** Activated HA-specific CD8<sup>+</sup> T cells were analyzed prior to their adoptive transfer for the expression of CD103 expression. One experiment, representative of 9, is shown. **(B)** Number of CD69<sup>+</sup>CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells (left) and frequency of MHC-II-expressing microglia (right) in the hypothalamus (black) and cerebellum (grey) of Orex-HA, on day 30 post-transfer. Each data point (n=10) is from 1 to 3 pooled Orex-HA mice. The results are from 3 independent experiments and were analyzed using the paired Student t-test. \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .



**Figure S2. Flow cytometry analyses of autoreactive CD8<sup>+</sup> T cells from the hypothalamus of Orex-HA mice confirm their tissue-resident phenotype.**

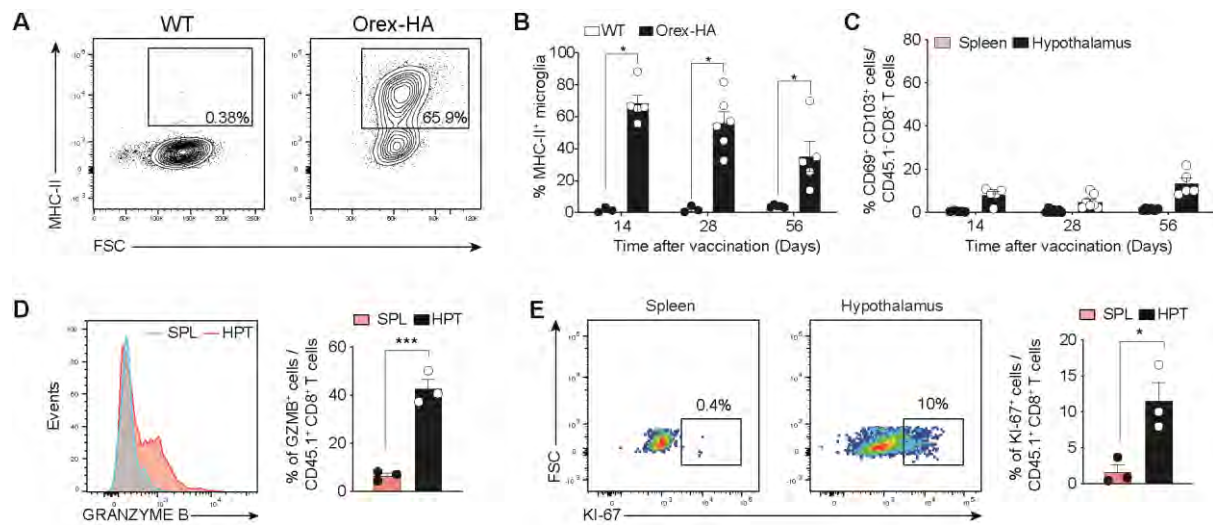
Representative histograms and the quantification of the geometric mean fluorescent intensity (gMFI) of CXCR6, CXCR3, CD49A, Ly-6C, EOMES and T-BET in transferred CD8<sup>+</sup> T cells from the spleen and hypothalamus of Orex-HA mice. Each data point (n=7-14) is from 2 to 14 pooled Orex-HA mice. The results are from 3 to 4 independent experiments and were analyzed using the Mann-Whitney's test. \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



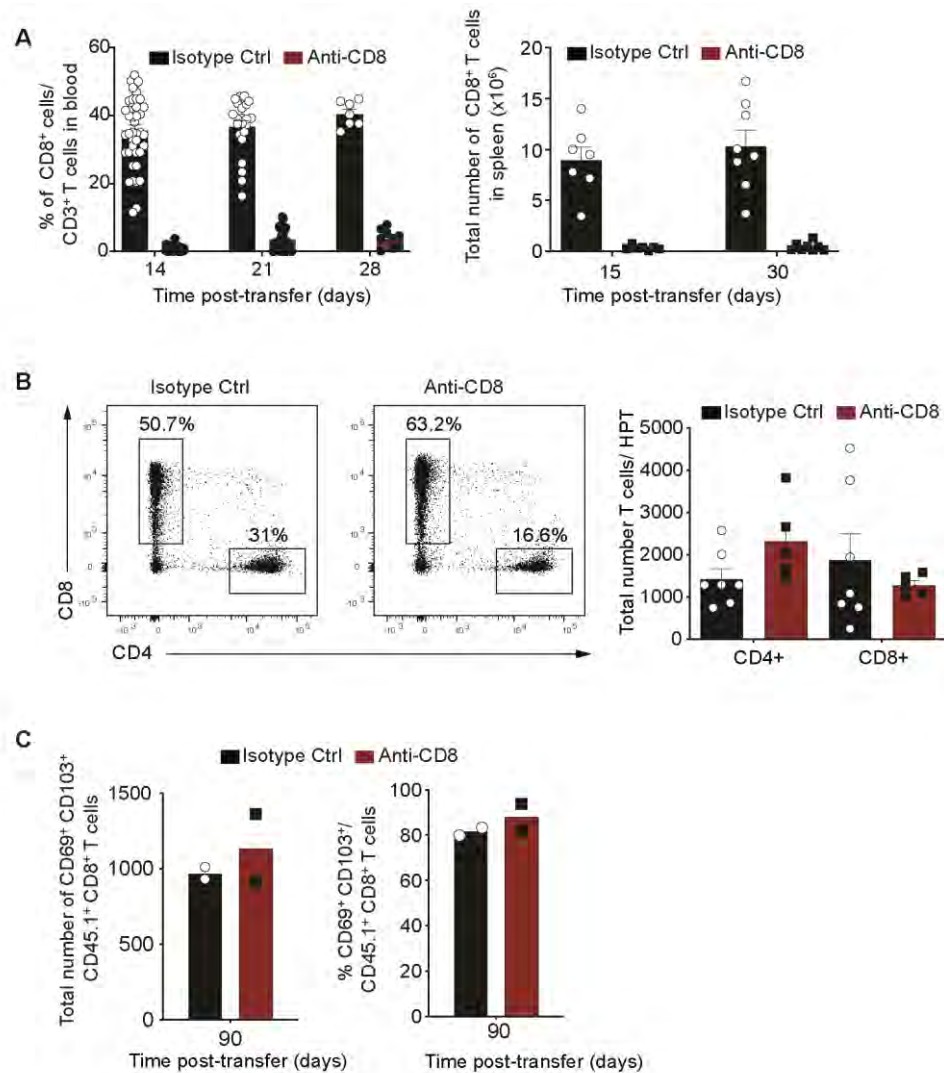


### **Figure S3. Heterogeneity of autoreactive tissue-resident CD8<sup>+</sup> T cells in the hypothalamus**

**(A)** Distribution of CD8<sup>+</sup> T cells from spleen and hypothalamus of Orex-HA mice in the nine clusters defined by scRNA-sequencing. **(B)** Plots of the GSEA for the brain TRM-, exhaustion- and IFN-signaling signatures in the transcriptome of the three hypothalamic clusters. **(C)** Violin plots showing expression of key brain TRM-signature genes in the 3 hypothalamic clusters. **(D)** Representative FACS plot (top) and quantification (bottom) of PD-1/TOX expression among transferred CD8<sup>+</sup> T cells from the hypothalamus of control IgG2b-treated Orex-HA mice, on day 30 (left). GRANZYME B (middle) and BST2 and Ly-6A (right) expression in the three transferred CD8<sup>+</sup> T cell populations identified based on PD-1 and TOX expression. Data are from 2 independent experiments involving 9 Orex-HA mice and were analyzed using a one-way ANOVA with Tukey's post hoc test. **(E)** Representative FACS plots (left) of GRANZYME B/PERFORIN co-expression within the three hypothalamic CD45.1<sup>+</sup> CD8<sup>+</sup> T cell populations of Orex-HA mice, on day 30 after activated CD8<sup>+</sup> T cell transfer. Frequency (right) of cells within the 3 hypothalamic CD45.1<sup>+</sup> CD8<sup>+</sup> T cell subsets expressing CD107A, GRANZYME A (GZMA), GRANZYME B (GZMB), PERFORIN, and GRANZYME B/ PERFORIN. **(F)** Representative FACS plots (*left*) of TNF/IFN $\gamma$  co-expression within the 3 hypothalamic CD45.1<sup>+</sup> CD8<sup>+</sup> T cell subsets of Orex-HA mice, on day 30 after activated CD8<sup>+</sup> T cell transfer. Frequency (right) of cells within the 3 hypothalamic CD45.1<sup>+</sup> CD8<sup>+</sup> T cell subsets expressing TNF, IFN $\gamma$ , and TNF/IFN $\gamma$ . Each data point (n=4) is from 2 pooled Orex-HA mice and results from one experiment. Statistical analyses in E and F were performed using two-way ANOVA with Tukey's post hoc test. \* $P < 0.05$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .

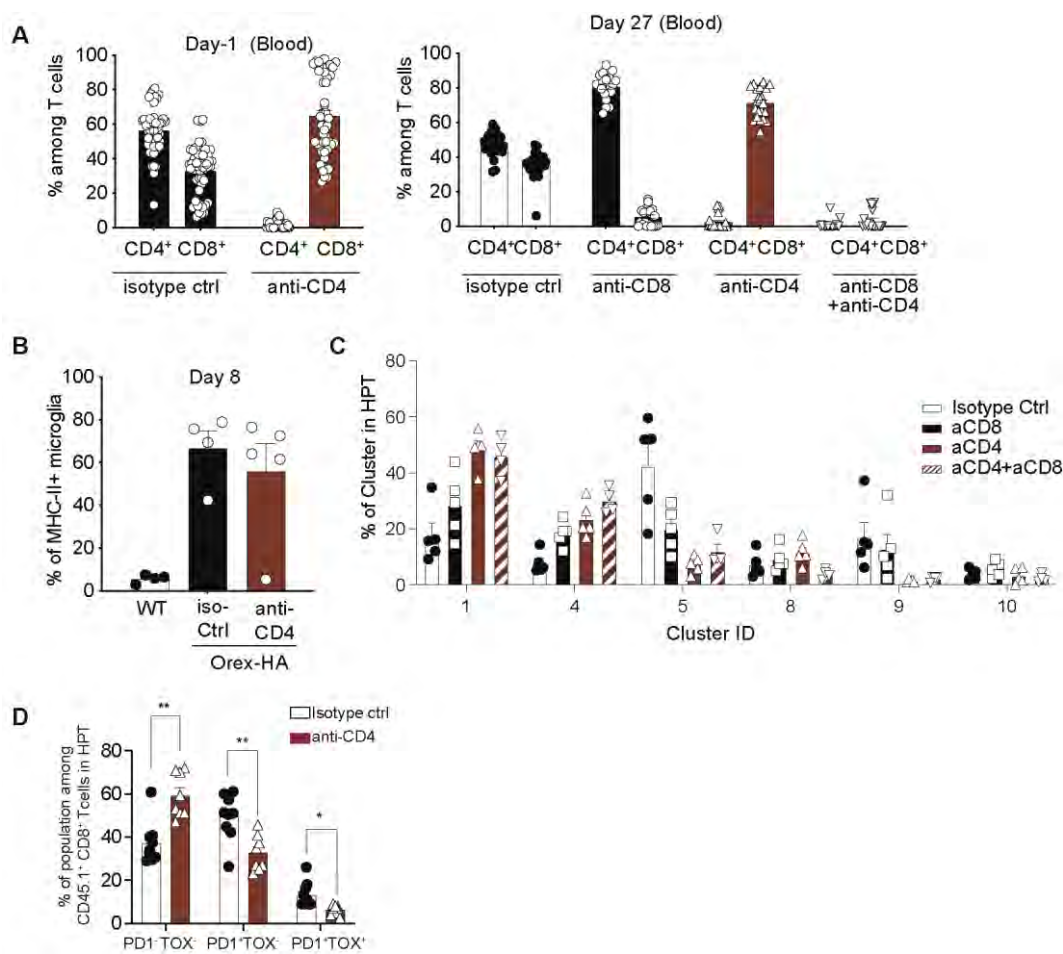


**Figure S4. Autoreactive tissue-resident CD8<sup>+</sup> T cells arise in the hypothalamus after in vivo priming.** (A) Representative histograms and (B) frequency of MHC-II expression on microglia (gated on live CD45.2<sup>int</sup>CD11b<sup>int</sup>LY-6c<sup>-</sup> cells) in the hypothalamus of Orex-HA and littermate control mice. Each data point (n=3-6) is from 2-18 pooled hypothalami and data are from 2 (day 56) or 3 (days 14 and 28) independent experiments. (C) Proportion of endogenous (CD45.1<sup>-</sup>) CD8<sup>+</sup> T cells expressing CD69 and CD103 in the spleen and hypothalamus of Orex-HA mice at different time-points post-vaccination. (D) Representative histograms and frequency of GRANZYME B-expressing transferred CD8<sup>+</sup> T cells in the spleen and hypothalamus of Orex-HA mice on day 28. (E) Representative FACS plots and frequency of KI-67-expressing transferred CD8<sup>+</sup> T cells in the spleen and hypothalamus on day 28 in one experiment involving 5-6 pooled Orex-HA mice per data point (n=3). Statistical analyses were performed with multiple Mann-Whitney test with Benjamini-Hochberg correction (B) and Student t-test (D,E). \**P* < 0.05, and \*\*\**P* < 0.001.



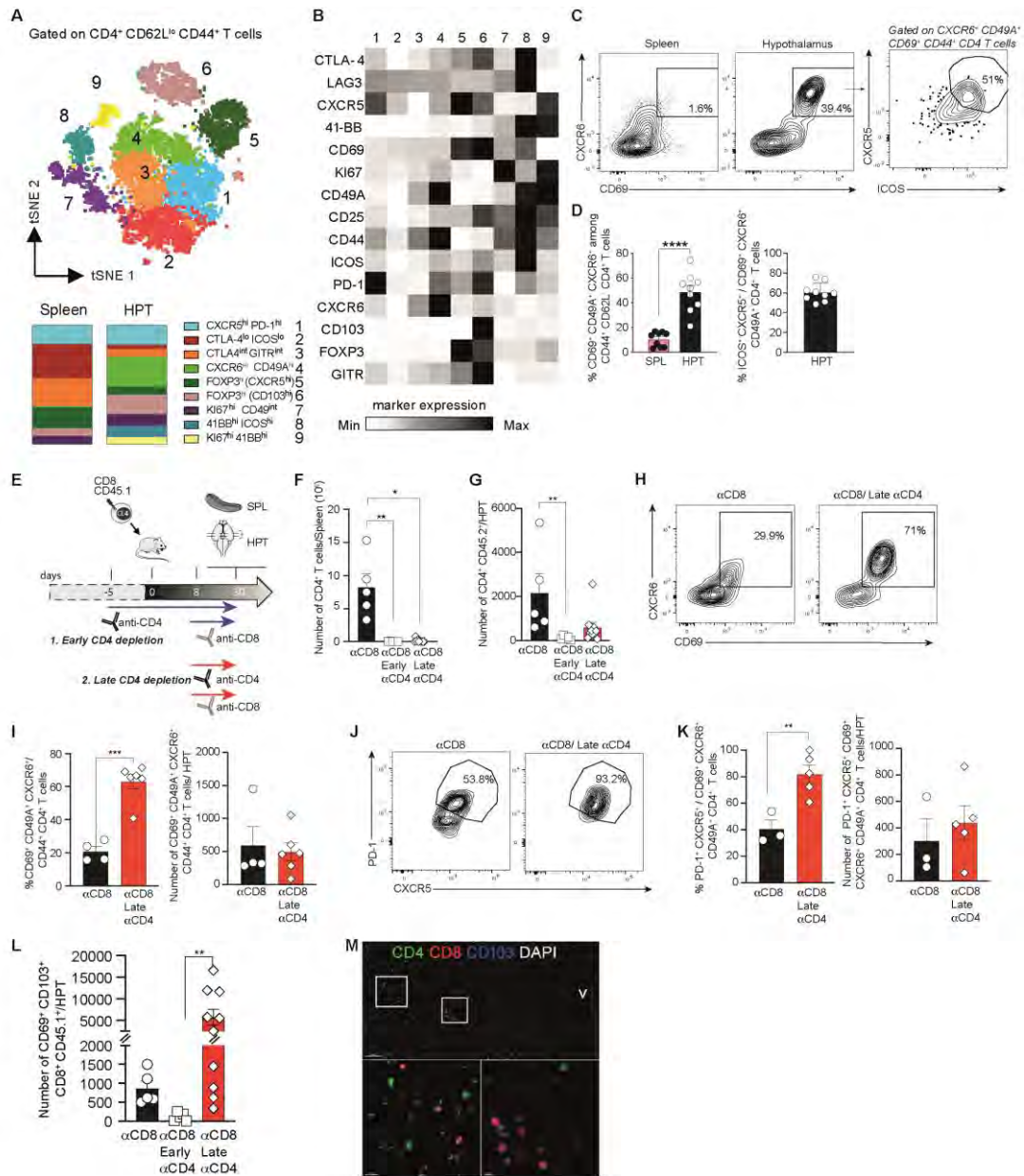
**Figure S5. Loss of hypothalamic orexinergic neurons is not dependent on circulating CD8<sup>+</sup> T cells.** (A) Frequency of blood T cells expressing CD8 (left) and numbers of endogenous (CD45.1<sup>+</sup>) CD8<sup>+</sup> T cells in the spleen (right), at different time-points after injection of anti-CD8 or isotype control antibodies in 2 to 3 independent experiments. Blood: data points represent individual mice (n ranging from 7 at day 28 to 43 at day 14). Spleen: D15: 7 mice per group; D30: 8 mice per group. (B) Representative dot-plots and absolute numbers of endogenous T cells in the hypothalamus of Orex-HA mice treated with either anti-CD8 or control antibodies, on day 30. Each data point (n=5-7) is from 2-pooled hypothalami and the data originate from 2 independent experiments. (C) Absolute numbers (left) and

frequency (right) of CD69<sup>+</sup> CD103<sup>+</sup> transferred CD8<sup>+</sup> T cells on day 90 in the hypothalamus of Orex-HA mice treated with anti-CD8 or control antibody. Each data point (n= 2) is from 2-pooled mice in one experiment.



**Figure S6. Tissue-resident CD8<sup>+</sup> T cell persistence and their functional properties in the hypothalamus are dependent on CD4<sup>+</sup> T cells and are required for sustained neuronal loss.** (A) Frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (gated on live TCRβ<sup>+</sup> cells) analyzed at D-1 (left panel) in the blood of mice treated with isotype control (Ctrl n=50) or anti-CD4 mAb (n=47); or analyzed at D+27 (right panel) in the blood of mice treated with isotype control (n=25), anti-CD8 mAb (n=21), anti-CD4 mAb (n=22) or both anti-CD4 and anti-CD8 mAb (n=20). Data are from 3 independent experiments. (B) Frequency of MHC-II expressing microglia on day 8 in the hypothalamus of littermate control mice or Orex-HA mice treated with anti-CD4 or control antibodies (n=4-5 per group) from 2 independent experiments. (C)

Frequency of the hypothalamic clusters in relation to the treatment group. Data are from one experiment involving 4-5 mice per group. **(D)** Comparison of the 3 subsets of transferred CD8<sup>+</sup> T cells defined based on PD-1 and TOX expression in the hypothalamus of Orex-HA mice treated with depleting anti-CD4 or control antibodies, on day 30. Data are from 2 independent experiments involving 8-9 individual mice/group. The data from the control group are also shown in Figure S3D. The data were analyzed using one-way ANOVA (B), or Student t-test with Bonferroni correction for multiple testing (D). \* $P < 0.05$ , and \*\* $P < 0.01$ .

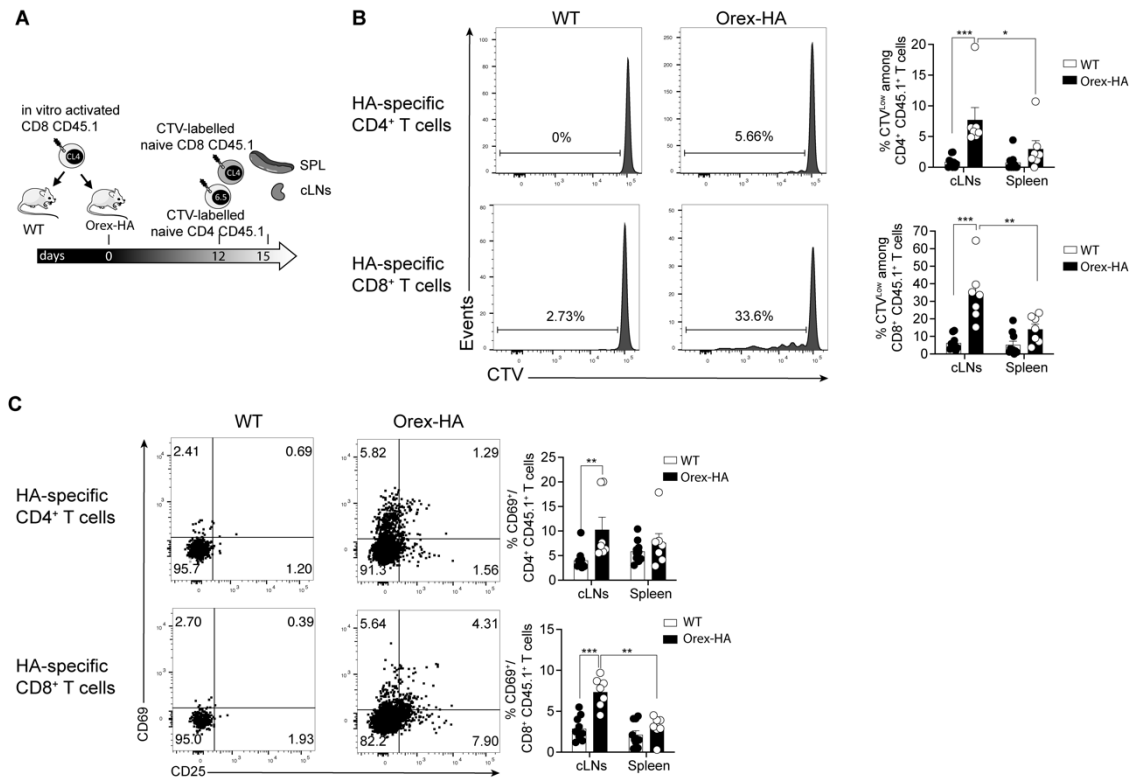


**Figure S7. Characterization of hypothalamic CD4<sup>+</sup> T cells involved in sustaining tissue-resident CD8<sup>+</sup> T cells.** (A) tSNE-graph with the cluster overlay of CD44<sup>+</sup> CD4<sup>+</sup> T cells from the hypothalamus and the spleen analysed by flow cytometry on day 30 post-transfer (top) and their relative representation in the two tissues (bottom). (B) Heatmap of the expression of key markers in the 9 identified clusters. (C, D) Representative FACS plots on day 30 (C) and frequency (D) of CD69<sup>+</sup> CXCR6<sup>+</sup> among CD44<sup>+</sup> CD62L<sup>lo</sup> CD4<sup>+</sup> T cells in the spleen and hypothalamus of Orexin-HA mice and the expression of CXCR5<sup>+</sup> and ICOS<sup>+</sup> by this

hypothalamic subset. Results are from 9 individual mice from 2 independent experiments. **(E)** Schematic experimental design: Orex-HA mice were assigned to one of 3 groups: 1. treatment with depleting anti-CD4 mAb starting 5 days prior to HA-specific CD8<sup>+</sup> T cell transfer ('early depletion'), as described in Fig. 7; 2. treatment with depleting anti-CD4 mAb from day 8 onwards ('late depletion'); 3. treatment with an isotype control antibody. In addition, from day 8 onwards, all 3 groups of mice received depleting anti-CD8 mAb. Absolute numbers of CD4<sup>+</sup> T cells in the spleen **(F)** and hypothalamus **(G)** at day 30 in the 3 groups of Orex-HA mice (no CD4 depletion; early CD4 depletion; late CD4 depletion; 5 to 10 mice per group) from 2 to 3 independent experiments. **(H)** Representative FACS plots of CD69<sup>+</sup> CXCR6<sup>+</sup> among CD44<sup>+</sup> CD62L<sup>-</sup> CD4<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with anti-CD8 mAb and isotype control or anti-CD4 mAb (late depletion), on day 30. **(I)** Frequency (*left*) or absolute numbers (*right*) of CD69<sup>+</sup> CXCR6<sup>+</sup> CD49A<sup>+</sup> CD44<sup>+</sup> CD62L<sup>-</sup> CD4<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with anti-CD8 mAb and control antibodies or anti-CD4 mAb (late depletion) in 1 to 4 pooled mice per data point (n=4-6) from 3 independent experiments. **(J)** Representative FACS plots of PD-1 and CXCR5 expression among CD69<sup>+</sup> CXCR6<sup>+</sup> CD49A<sup>+</sup> CD44<sup>+</sup> CD62L<sup>-</sup> CD4<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with anti-CD8 mAb and control antibodies or anti-CD4 mAb (late depletion), on day 30. **(K)** Frequency (*left*) and absolute numbers (*right*) of CXCR5<sup>+</sup> PD-1<sup>+</sup> CD69<sup>+</sup> CXCR6<sup>+</sup> CD49A<sup>+</sup> CD4<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with anti-CD8 mAb and control antibodies or anti-CD4 mAb (late depletion) in 3 to 5 individual mice per group from 2 independent experiments. **(L)** Absolute numbers of transferred (CD45.1<sup>+</sup>) CD69<sup>+</sup> CD103<sup>+</sup> CD8<sup>+</sup> T cells in the hypothalamus of Orex-HA mice treated with anti-CD8 mAb and control antibodies or anti-CD4 mAb (late and early depletion), on day 30, in 5 to 10 mice per group from 2 to 3 independent experiments. **(M)** Immunofluorescence microscopy identifying CD4<sup>+</sup> cells in the vicinity of tissue-resident CD8<sup>+</sup> T cells in the



hypothalamus of Orex-HA mice, on day 30 (top; scale bar: 100 $\mu$ m). The two boxed regions are shown at a higher magnification (bottom; scale bar: 20 $\mu$ m); V, blood vessel. Statistical analyses were performed using Mann-Whitney (D), Student t-test (I, K) and Kruskal-Wallis' test with Dunn's correction (F, G, L). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .



**Figure S8. Antigen-specific T cell activation during disease course suggests that neuronal antigens drain in cervical LNs. (A)** Schematic experimental design: Orex-HA and littermate control (WT) mice were adoptively transferred i.v. with HA-specific effector CD8<sup>+</sup> T cells. On day 12, recipient mice received CTV-labelled naïve HA-specific CD45.1<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells from 6.5 and CL4-TCR transgenic mice, respectively. Proliferation and activation of the naïve T cells were analysed in cervical lymph nodes (cLNs) and spleen (SPL) three days later. **(B)** Representative FACS plots (*left*) and frequency (*right*) of CTV<sup>low</sup> antigen-specific CD4<sup>+</sup> (*top*) and CD8<sup>+</sup> (*bottom*) T cells in the cLNs and spleen of Orex-HA and WT mice. **(C)** Representative FACS plots (*left*) and frequency (*right*) of CD69<sup>+</sup> antigen-specific CD4<sup>+</sup> (*top*) and CD8<sup>+</sup> (*bottom*) T cells in the cLNs and spleen of Orex-HA and WT mice. Results are from in 7 to 10 mice per group from 2 independent experiments. Statistical analyses were performed using multiple Mann-Whitney's test with Benjamini-Hochberg correction (B,C). \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## Supplementary tables:

**Table S1. Patients' characteristics**

<b>Patient</b>	<b>Diagnosis</b>	<b>Age</b>	<b>Sex</b>
1	Limbic/brain stem encephalitis associated with anti-Ma2 antibodies	77	F
2	Paraneoplastic cerebellar degeneration associated with anti-Yo antibodies	66	M
3	Limbic/brain stem encephalitis associated with anti-Ma2 antibodies	77	F
4	Paraneoplastic encephalomyelitis associated with anti-Hu antibodies	83	M
5	Paraneoplastic encephalitis associated with anti-Ri antibodies	59	F
6	Paraneoplastic encephalomyelitis associated with anti-Hu antibodies	72	M
7	Paraneoplastic encephalomyelitis associated with anti-Hu antibodies	74	M
8	Paraneoplastic limbic encephalitis associated with anti-Ma2 antibodies	72	M
9	Paraneoplastic neuronopathy associated with anti-Hu antibodies	71	M
10	Paraneoplastic limbic encephalitis associated with anti-Hu antibodies	65	M

**Table S2. Monoclonal antibodies used for flow cytometry**

<b>Antibody</b>	<b>Company</b>	<b>Clone</b>	<b>Origin</b>
□4β7 (LPAM1)	BD Pharmingen	DATK32	Rat
BCL-2	eBioscience	10C4	Rat
BST-2	BD Pharmingen	927	Rat
CD103	BD Pharmingen	M290	Rat
CD107a	BD Pharmingen	1D4B	Rat
CD11a (LFA-1)	BioLegend	M17/4	Rat
CD11b	eBiosciences	M1-70	Rat
CD11c	eBiosciences	N418	Hamster
CD127	BD Pharmingen	SB/199	Rat
CD137 (41-BB)	eBiosciences	17B5	Hamster
CD152 (CTLA4)	BD Pharmingen	UC10-4F10-11	Hamster
CD183 (CXCR3)	eBiosciences	CXCR3-173	Hamster
CD185 (CXCR5)	Miltenyi	REA215	Rat
CD186 (CXCR6)	Miltenyi	REA1193	Rat
CD186 (CXCR6)	BioLegend	SA051D1	Rat
CD195 (CCR5)	eBiosciences	HM-CCR5 (7A4)	Hamster
CD195 (CCR7)	BD Pharmingen	4B12	Rat
CD19	BD Pharmingen	1D3	Rat
CD25	BioLegend	PC61	Rat
CD278 (ICOS)	BD Pharmingen	7E.17G9	Rat
CD279 (PD-1)	BioLegend	29F.1A12	Rat
CD357 (GITR)	BD Pharmingen	DTA-1	Rat
CD4	BD Pharmingen	RM4-5	Rat
CD44	eBiosciences	IM7	Rat
CD45.1	BD Pharmingen	A20	Rat
CD45.2	BioLegend	104	Rat
CD49a	BD Pharmingen	Ha31/8	Hamster
CD49d	BD Pharmingen	R1-2	Rat
CD62L	BD Pharmingen	MEL-14	Rat
CD69	BD Pharmingen	H1.2F3	Hamster
CD8a	BD Pharmingen	53-6.5	Rat

CD8b	BD Pharmingen	H35-17.2	Rat
EOMES	eBiosciences	Dan11mag	Rat
FOXP3	eBiosciences	FJK-16s	Rat
GM-CSF	BD Pharmingen	MP1-22E9	Rat
GRANZYME A	eBiosciences	GzA-3G8.5	Rat
GRANZYME B	BioLegend	QA16A02	Rat
IFN- $\gamma$	BD Pharmingen	XMG1.2	Rat
IgG2b	BD Pharmingen	R35-38	Rat
(Isotype Control)			
IL-10	eBiosciences	JES5-16E3	Rat
IL-17A	BD Pharmingen	TC11-18H10	Rat
Ki-67	BD Pharmingen	B56	Rat
Ki-67	eBiosciences	SolA15	Rat
LAG-3	eBioscience	C9B7W	Rat
Ly6a (Sca-1)	BioLegend	D7	Rat
Ly6c	BioLegend	HK1.4	Rat
Ly108 (Slamf6)	BD Pharmingen	13G-3	Rat
MHC-II	BioLegend	M5/114.15.2	Rat
NK-1.1	BioLegend	PK136	Rat
PERFORIN	eBiosciences	eBioOMAK-D	Rat
P2X7R	BioLegend	1F11	Rat
T-Bet	BD Pharmingen	4B10	Rat
TCR $\beta$	BD Pharmingen	H57-597	Hamster
TCR V $\beta$ 8.1, 8.2	BD Pharmingen	MR5-2	Rat
Thy1.2	BD Pharmingen	53-2.1	Rat
TIM-3	BD Pharmingen	SD12/TIM-3	Rat
TOX	Miltenyi	REA473	Rat
TNF	BD Pharmingen	MP6-XT22	Rat

**Table S3. Antibodies used for immunohistology**

<b>Antibody</b>	<b>Origin</b>	<b>Target</b>	<b>Dilution</b>	<b>Reactive fluorophore</b>	<b>Company</b>
Orexin	Rabbit	Orexin A	1:20000 1:6000 1:2000	Cy5 Opal650 DAB	Phoenix Pharmaceuticals #003-30
CD3	Rabbit	Epsilon chain of human CD3	1:500	Opal690	Neomarkers #RM9107-S
CD4	Rabbit	Human CD4	1:250	Opal 520	Cell Signaling # 48274
CD4	Rabbit	Mouse CD4	1:1000	Opal 480	Sino Biological #50143-MO8H
CD8a	Rabbit	Mouse CD8 alpha	1:250 1:2000	Cy2 Opal570	Abcam #ab209775
CD8	Mouse	Human CD8 $\alpha$	1:500	Opal480	DAKO #M7103
GRZMB	Mouse	Human Granzyme B	1:100	Opal520	Neomarkers #MS-1157-S1
GRZMB	Rabbit	Human/mouse granzyme B	1:25	Cy3	Abcam ab4059
CD103	Rabbit	Human Integrin $\alpha$ E	1:5000	Opal570	Abcam #ab129202
CD103	Goat	Mouse Integrin $\alpha$ E/CD103	1:250	Cy3	R&D #AF1990
PD-1	Rabbit	Human PD1	1:150	Opal 570	Abcam #ab137132
PD-1	Rabbit	Human/Mouse PD1	1:50	Cy3	Proteintech #18106-1-AP
CD49a	Sheep	Human Integrin $\alpha$ 1/ CD49a	1:250	Cy2	Thermo Fisher #PA5-47763
CD69	Rabbit	Human CD69	1/50	Goat anti- rabbit AF488	Sigma #HPA0505525
BCL2	Mouse	Human BCL2	1/50	Goat anti- mouse IgG1 AF555	Dako Ref:M0887

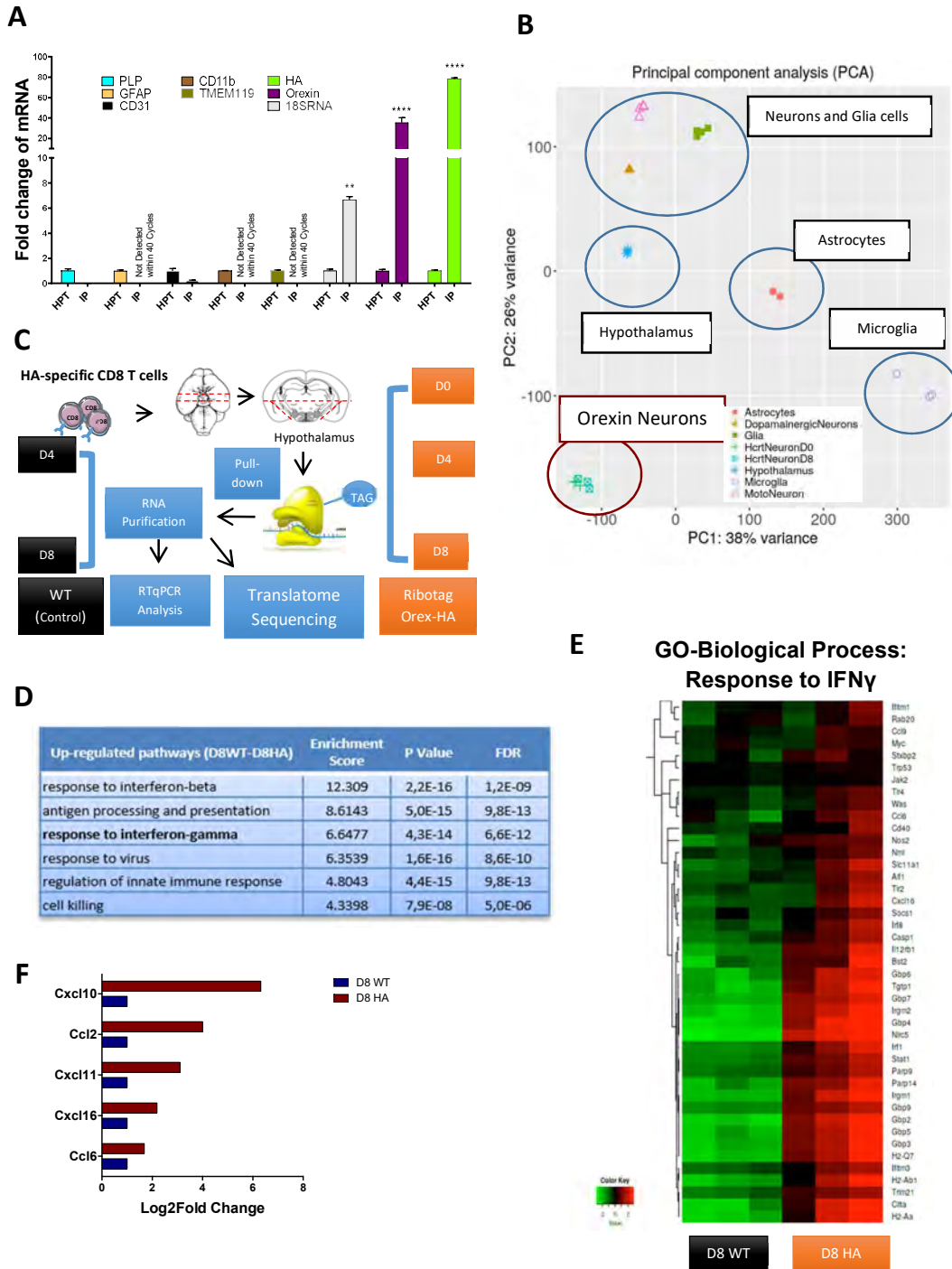
TOX	Rat	TOX	1/20	Anti-rat HRP +amplification Opal 520	ThermoFisher Ref:14-6502-82 Clone TXRX10
GRZMB	Mouse	Human GrzmB	1/20	Anti mouse HRP +amplification Opal 570	Monosan Ref 7029 Clone GrB-7
CD8	Mouse	Cytoplasmic domain of human CD8 $\alpha$	1:20	Goat anti- mouse IgG1 AF 647	DAKO #M7103
CD8a	Mouse	Human CD8	1/50	Goat-anti mouse IgG2b AF647	Thermo #MA1-80231 Clone 4B11



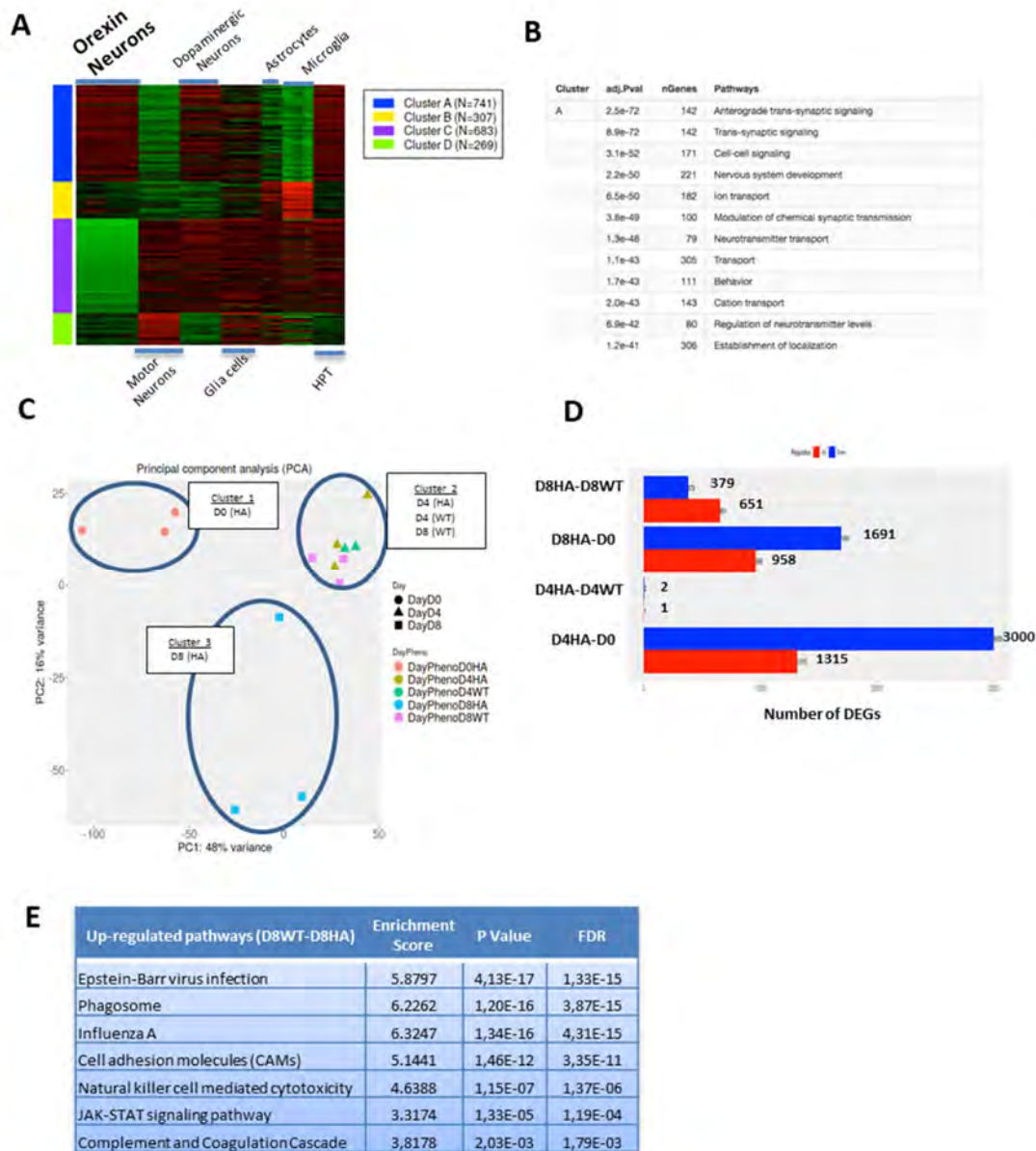
## 2.3 IFN $\gamma$ signaling is involved in neuronal destruction and persistence of CD8 T<sub>RM</sub> cells in the hypothalamus.

### 2.3.1 Translational profiling of orexin neurons under CD8 T cell attack reveals upregulation of IFN $\gamma$ responsive genes in an autoantigen-dependent manner

We aimed to analyze translational changes in orexin neurons which occur upon interaction with autoreactive CD8 T cells, in order to elucidate the pathogenic mechanism involved in the neuronal loss. To this end, we made use of a method used for translational profiling via the expression of a tagged Ribosome subunit, which makes it possible to immunoprecipitate mRNA that is in the course of translation. First of all, we validated the method by comparison of the immunoprecipitated mRNA (IP) with that from the whole hypothalamus (HPT) before immunoprecipitation. 18SrRNA transcripts were 7.6-fold enriched – proving that ribosomes were indeed selectively captured by the IP. Furthermore, we could detect little or no mRNA transcripts of endothelial cells (CD31), astrocytes (GFAP), or microglia/border-associated macrophages (TMEM119, CD11b). On the contrary, orexin-neuron-specific transcripts were more than 40-fold enriched (**Fig 2.1A**). We then proceeded to generate RNA libraries from immunoprecipitated mRNA for subsequent RNA sequencing after different days of the injection of effector HA-specific CD8 T cells (**Fig 2.1B**). We compared our dataset with public data sets from other cell types of the CNS and found that orexin neurons clustered quite distinctly away from other CNS resident cells. The pathways that accounted for this difference were involved in trans-synaptic signaling, neurotransmitter transport, and behavior – pathways that are accepted to be specific to orexin neurons (**Fig 2.1C and Supp. Fig 2.1A, B**). We used mice which expressed the flagged ribosome, but not HA in orexin neurons, to dissociate bystander effects from pathways that are induced in an antigen-specific manner. Surprisingly, it was only on day 8 (but not on day 4) that we were able to observe a substantial number of differentially expressed genes (DEGs) between the translational profiles of orexin neurons from HA-expressing (HA) and non-HA-expressing (WT) mice. The most highly enriched pathways included the response to IFN $\beta$  and IFN $\gamma$  (**Fig 2.1D and Supp. Fig 2.1C-E**). Within the pathway in response to IFN $\gamma$ , STAT1 and several chemokines (*CCL2*, *CXCL16*, *CCL6*, *CXCL10*) were upregulated in orexin neurons from HA mice (**Fig 2.1E, F**). Besides, an antigen processing and presentation pathway was upregulated including several H2 molecules of the MHC-I complex. In sum, these data suggest that several translational changes in immunoprecipitated mRNA which resemble orexin neurons are induced in an antigen-specific manner only from day 8 onwards. The pathways suggest that the response to IFN $\gamma$  triggers the expression of chemokines and the enhanced processing and presentation of antigens.



**Figure 2.1 Translational profiling of orexin neurons after adoptive transfer of CD8 T cells** (A) RTqPCR analysis of cell type-specific genes (*PLP*, *GFAP*, *CD11b*, *CD31*, *TMEM119*, *18SRNA*, *orexin*, and *HA*) comparing the mRNA of the immunoprecipitated Ribosome (IP) with the mRNA of the whole hypothalamus (HPT); statistics from Student’s t-test by comparing at least three mice per group performed within one to three independent experiments. (B) Principal component analysis of our data set compared to public data set of brain-specific cell populations (obtained from iDEP91). (C) Workflow for the translation profiling of orexin neurons before (D0) or after different days of Tc1 transfer (D) Table indicates details for six chosen among the TOP20 upregulated GO Biological process pathways of the group D8HA compared to D8 WT detailing the enrichment score, p-Value and FDR. (E) Heat map of the TOP 40 differentially expressed genes within the GO-Biological process “Response to IFN $\gamma$ ”. (F) Fold-change upregulation of five chemokines upregulated in the group D8HA compared to D8WT.



**Supplementary Figure 2.1 Translational profiling of orexin neurons after adoptive transfer of CD8 T cells**

**(A)** Heat map of the cluster analysis of the comparison between our data set and public data set of CNS resident cells. **(B)** GO Biological pathways associated with the cluster upregulated in orexin neurons. **(C)** Principal component analysis of orexin neurons after different days of effector CD8 T cell transfer. **(D)** Number of Differential expressed genes of the comparisons we analyzed. **(E)** The table indicates details for seven chosen among the TOP20 upregulated KEGG pathways of the group D8HA compared to D8 WT detailing the enrichment score, p-value, and FDR.

### 2.3.2 IFN $\gamma$ deficiency prevents neuronal destruction in a CNS autoimmune model

Based on the IFN $\gamma$  response pathway revealed by the translatomic profiling data and the abrogation of orexin neuron loss by injection of IFN $\gamma$  deficient CD8 T cells in the vaccine model, we hypothesized that IFN $\gamma$  might be important for orexin neuron loss. We now aimed to understand the mechanism involved. For this, we injected *in vitro* activated HA-specific CD8 T cells sufficient (IFN $\gamma^{+/+}$ ) or deficient (IFN $\gamma^{-/-}$ ) for IFN $\gamma$  into Orex-HA mice or littermate control mice (WT) and analyzed the impact on orexin neuron loss (**Fig 2.2A**).

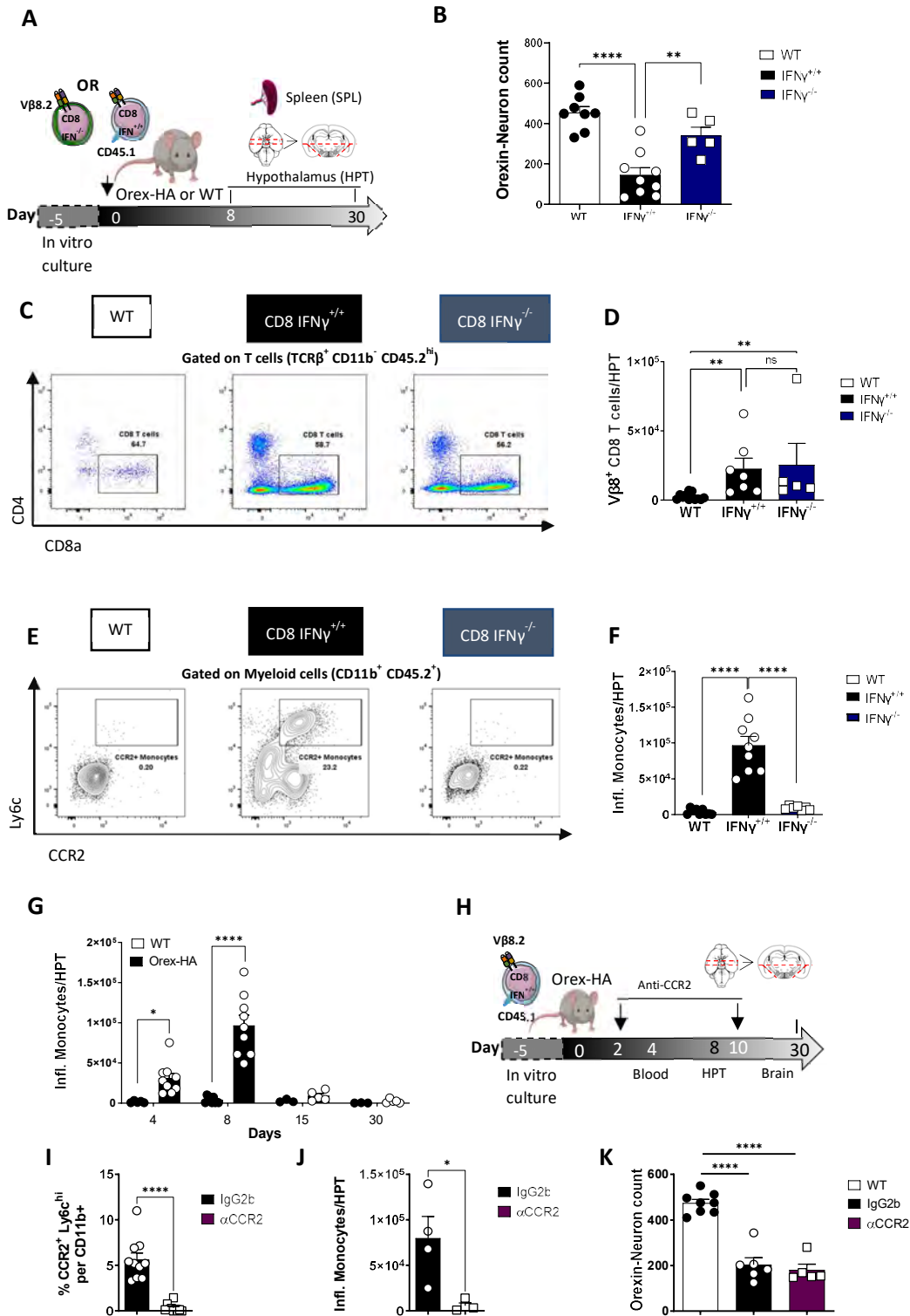
On day 30, there was a significant neuronal loss induced by IFN $\gamma^{+/+}$  CD8 T cells in Orex-HA compared to WT mice, where the neo-antigen is not expressed in orexin neurons. However, the neuronal loss was significantly reduced upon the injection of IFN $\gamma^{-/-}$  CD8 T cells into Orex-HA mice (**Fig 2B**). IFN $\gamma$  is important for T cell transmigration over the blood-brain barrier (Sonar et al. 2017) by inducing ICAM-1 and VCAM-1 on brain endothelial cells. Therefore, we hypothesized that the observed phenotype might be due to reduced infiltration of IFN $\gamma^{-/-}$  CD8 T cells in the hypothalamus. Contrary to this assumption, V $\beta$ 8<sup>+</sup> CD8 T cells – which on average represent 83.42% of the transferred CD8 T cells (**Supp. Fig 2.2A**) – entered the hypothalamus of Orex-HA mice in similar numbers on day 8, regardless of their ability to produce IFN $\gamma$  (**Fig 2.2C, D**). We further analyzed the functionality of the effector CD8 T cells before the transfer, as the absence of IFN $\gamma$  during differentiation might have impacted pathogenic functions. Besides the lack of production of IFN $\gamma$  and a slight increase in the Granzyme B store, we found no differences in the expression of cytokines, activation markers, chemokine receptors, and integrins or transcription factors (**Supp. Fig 2.2B-D**).

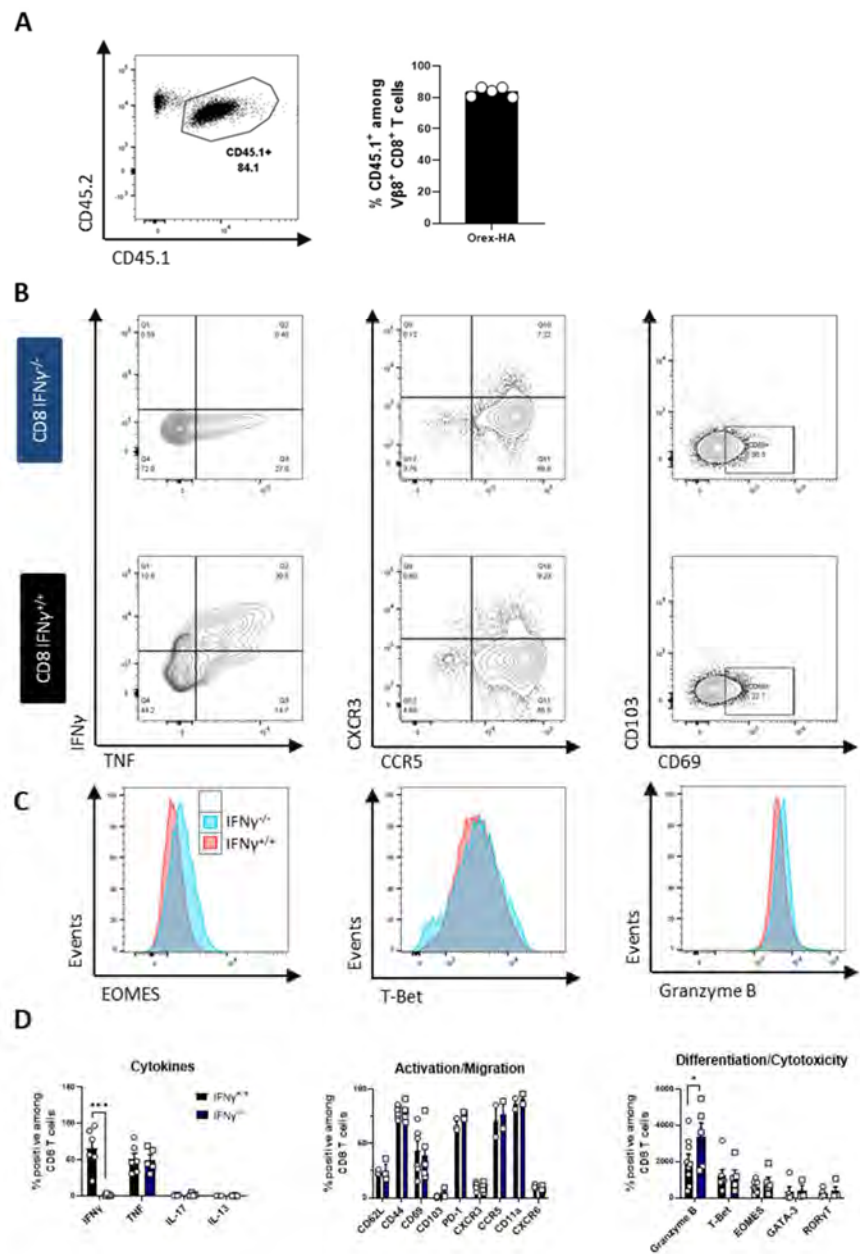
Di Liberto et al. demonstrated that IFN $\gamma$  signaling in neurons promotes their secretion of CCL2, thereby attracting CCR2-expressing monocytes to the brain, which strips the synapses of neurons, resulting in their loss (Di Liberto et al. 2018). We evaluated whether a similar mechanism might be at play in our model. We confirmed the infiltration of  $96.664 \pm 12.645$  monocytes on day 8 in the hypothalamus induced by IFN $\gamma^{+/+}$  CD8 T cells, which was absent upon transfer of IFN $\gamma^{-/-}$  CD8 T cells in Orex-HA mice (**Fig 2.2E, F**).

To assess whether the lack of infiltrating monocytes is responsible for the prevention of the orexin neuron loss, we aimed to prevent their CNS infiltration in the context of the transfer of HA-specific IFN $\gamma^{+/+}$  CD8 T cells. By performing kinetic experiments, we found that monocytes infiltrated the hypothalamus of Orex-HA mice from day 4 onwards, reaching a maximum on day 8, while no differences were observed in Orex-HA compared to WT mice from day 15 onwards (**Fig 2.2G**). Therefore, we started to deplete monocytes from day 2 onwards until day

10 (**Fig 2.2H**). The frequency of monocytes in the blood 48 hours post-anti-CCR2 injection confirmed an efficient depletion, and subsequently few monocytes were found in the hypothalamus on day 8 (**Fig 2.2I+J**). Despite this, orexin neuron loss was not reversed by the depletion of monocytes (**Fig 2.2K**). All things considered, HA-specific  $\text{IFN}\gamma^{-/-}$  CD8 T cells infiltrate the hypothalamus but are not able to induce neuronal loss. This is not due to the lack of monocytes attraction to the hypothalamus, but might be due to the lack of antigen-mediated killing of orexin neurons by CD8 T cells, as  $\text{IFN}\gamma$  is crucial to induce MHC-I expression on neurons (Neumann et al. 1997). Furthermore,  $\text{IFN}\gamma$ -mediated activation microglia may be at play, which could release neurotoxic molecules.

**Figure 2.2  $\text{IFN}\gamma$  deficiency prevents neuronal destruction in a CNS autoimmune model** (A) Scheme showing the experimental design: Orex-HA and littermate control (WT) mice ( $\text{CD45.1}^{-}$ ) were adoptively transferred i.v. with HA-specific effector CD8 T cells ( $\text{V}\beta 8.2^{+}$ ;  $\text{CD45.1}^{+}$ ) sufficient ( $\text{IFN}\gamma^{+/+}$ ) or deficient ( $\text{IFN}\gamma^{-/-}$ ) for  $\text{IFN}\gamma$  after five days of *in vitro* activation. (B) On day 30 post-transfer, orexin A<sup>+</sup> neurons were quantified by Immunohistochemistry throughout the hypothalamus. The data are from 5 to 9 mice per group in two independent experiments. (C+D) Representative FACS plots (C) on day 8 and number (D) of  $\text{CD45.2}^{+}\text{CD11b}^{-}\text{TCR}\beta^{+}\text{CD8}^{+}\text{V}\beta 8.2^{+}$  cells in the hypothalamus of Orex-HA and WT mice receiving  $\text{IFN}\gamma^{+/+}$  CD8 T cells or Orex-HA mice receiving  $\text{IFN}\gamma^{-/-}$  CD8 T cells. Data from 5 to 9 mice in 2 independent experiments. (E) Representative FACS plot of CCR2 expression among myeloid cells (gated on living  $\text{CD45.2}^{+}\text{CD11b}^{+}$  cells) in the hypothalamus. (F) Quantification of inflammatory monocytes ( $\text{CD45.2}^{\text{int}}\text{CD11b}^{\text{hi}}\text{Ly-6C}^{+}$  cells) in the hypothalamus at day 8. The data are from 8 to 9 mice in 2 independent experiments. (G) Quantification of inflammatory monocytes in the hypothalamus of Orex-HA and WT mice following the transfer of  $\text{IFN}\gamma^{+/+}$  CD8 T cells. The data are from 3 to 9 mice in 2 to 3 independent experiments. The day 8 data are the same as those presented in F. (H) Schematic experimental design: Orex-HA mice were treated daily with depleting anti-CCR2 monoclonal antibodies (MC21) starting from day 2 until day 10 post-transfer. (I) Percentage of inflammatory monocytes among myeloid cells in the blood on day 4 from 10 mice per group in two independent experiments. (J) Number of inflammatory monocytes in the hypothalamus of Orex-HA mice treated or not with the anti-CCR2 mAb on day 8 from 4 mice per group in one experiment. (K) Quantification of orexin A<sup>+</sup> neurons in the hypothalamus of Orex-HA mice treated or not with the anti-CCR2 mAb on day 30 post-transfer. The data are from 5 to 8 mice per group in one experiment. Statistical analyses were performed using one-way ANOVA Tukey’s post hoc test (B, D, F, K) and Mann-Whitney test (I, J). For all the panels, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .





**Supplementary Figure 2.2** (A) Representative FACS plot and frequency of CD45.1 expression among Vβ8+ CD8 T cells in the hypothalamus. (B) Representative FACS plot of IFNγ and TNF, CXCR3 and CCR5 as well CD69 and CD103 expression among effector CD8 T cells on day 5 after *in vitro* culture. For the left panel, the cells were stimulated for 4hrs in the presence of PMA and Ionomycin. (C) Representative histograms of EOMES, T-bet, and Granzyme B expression among effector CD8 T cells on day 5 after *in vitro* culture. (H) Quantification of several cytokines, activation/migration, and intracellular proteins among effector CD8 T cells on day 5 after *in vitro* culture. Results were obtained from at least two independent experiments. Statistical analyses were performed using two-way ANOVA with Sidak’s post hoc test \* p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.



### 2.3.3 IFN $\gamma$ deficiency impairs CD8 T<sub>RM</sub> maintenance in the CNS

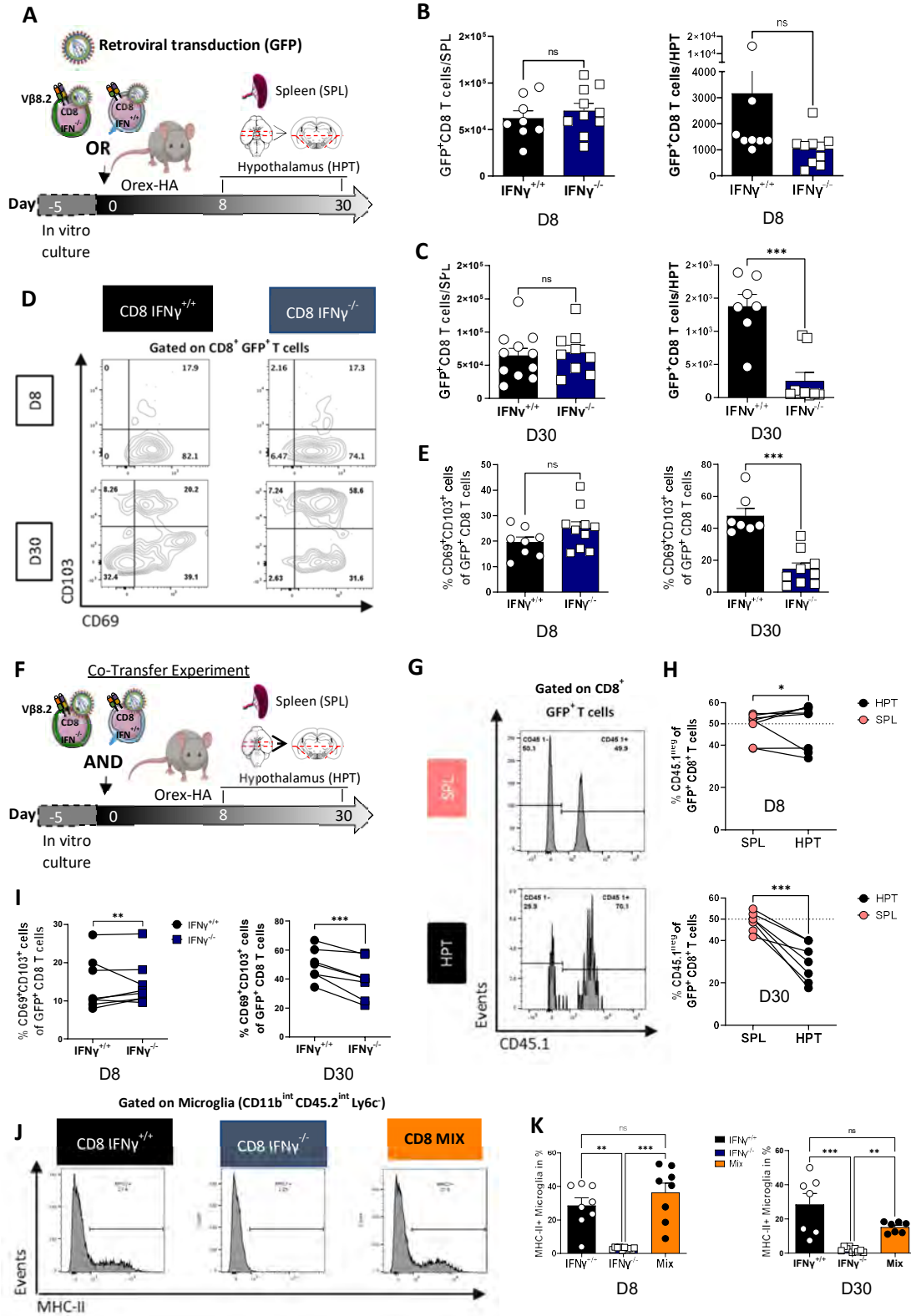
We next aimed to find out the reason for the reduced neuronal loss upon transfer of IFN $\gamma$ <sup>-/-</sup> CD8 T cells by analysis of the hypothalamus on day 30 where we performed our neuronal count. We also wanted to study the inflamed state of the hypothalamus and the phenotype of the transferred IFN $\gamma$ <sup>-/-</sup> CD8 T cells. The deficiency in IFN $\gamma$  might have resulted in a reduced recruitment of other lymphocytes or myeloid cells, which might have a pathogenic role in the orexinergic neuron destruction. Due to the lack of the congenic marker CD45.1 on IFN $\gamma$ <sup>-/-</sup> CD8 T cells, we transduced both IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> HA-specific CD8 T cells with a GFP-expressing retrovirus to trace them *in vivo* (**Fig 2.3A**). On day 8, IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> CD8 T cells were present in the spleen in similar numbers and, akin to the results above, CD8 T cells from both groups were able to seed the hypothalamus (**Fig 2.3B**). Nevertheless, on day 30, although their numbers did not differ from each other in the spleen, IFN $\gamma$ <sup>-/-</sup> CD8 T cells did not persist in the hypothalamus on day 30 compared to IFN $\gamma$ <sup>+/+</sup> CD8 T cells (**Fig 2.3C**). Our previous study provided evidence that in this autoimmune setting the vast majority of hypothalamus-infiltrating CD8 T cells adopted a T<sub>RM</sub> phenotype expressing CD69 and CD103 (Frieser et al. 2022). We, therefore, studied the phenotype of hypothalamus-infiltrating IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> CD8 T cells on day 8 and day 30. Although on day 8 the proportion of transferred CD8 T cells expressing CD69 and CD103 was similar in both groups, the proportion of transferred IFN $\gamma$ <sup>-/-</sup> CD8 T cells expressing those two T<sub>RM</sub> markers decreased on day 30 (**Fig 2.3D, E**). Therefore, IFN $\gamma$ <sup>-/-</sup> CD8 T cells appear to not persist in the CNS up to day 30, with impaired expression of T<sub>RM</sub> markers.

Studies of brain CD8 T<sub>RM</sub> cells provided evidence that local antigen re-encounter is essential for CD8 T<sub>RM</sub> formation in the brain (Schøller et al. 2019). As IFN $\gamma$  is important for antigen presentation by the induction of MHC-I on neurons, we hypothesized that IFN $\gamma$ <sup>-/-</sup> CD8 T cells could not efficiently differentiate into T<sub>RM</sub> cells, because they could not recognize HA in orexin neurons. To control for this and assess whether the lack of CNS persistence of IFN $\gamma$ <sup>-/-</sup> CD8 T cells is cell-intrinsic, we co-transferred IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> effector CD8 T cells in Orex-HA mice. The transferred T cells can be distinguished, as IFN $\gamma$ <sup>+/+</sup> but not IFN $\gamma$ <sup>-/-</sup> CD8 T cells express the congenic marker CD45.1 (**Fig 2.3F**). On day 8, a comparable number of IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> CD8 T cells seeded the hypothalamus. But on day 30, IFN $\gamma$ <sup>+/+</sup> CD8 T cells outnumbered the IFN $\gamma$ <sup>-/-</sup> counterpart in the hypothalamus. By contrast, the ratio in the spleen remained stable between the two groups over time similar to the ratio that was initially injected (**Fig 2.3G, H**). Furthermore, the expression of CD69 and CD103 on day 30 was lower on hypothalamus-infiltrating IFN $\gamma$ <sup>-/-</sup> as compared to IFN $\gamma$ <sup>+/+</sup> CD8 T cells (**Fig 2.3I**).

We also looked at MHC-II expression on microglia which could give us an idea of the capacity of antigen presentation in the brain. IFN $\gamma$ <sup>-/-</sup> CD8 T cells did not induce MHC-II expression on microglia. Nevertheless, the co-transfer resulted in MHC-II expression on microglia to the level observed upon injection of IFN $\gamma$ <sup>+/+</sup> CD8 T cells into Orex-HA mice (**Fig 2.3J, K**). With this experiment, we made the argument unlikely that the impaired persistence of IFN $\gamma$ <sup>-/-</sup> CD8 T cells were due to not re-encountering their antigen in the CNS. Collectively these data suggest that the lack of constant IFN $\gamma$  signaling might have a cell-intrinsic role in the maintenance of the CD8 T<sub>RM</sub> program. One could think that IFN $\gamma$  is secreted and recognized by the very same CD8 T cell, therefore creating an autocrine loop.

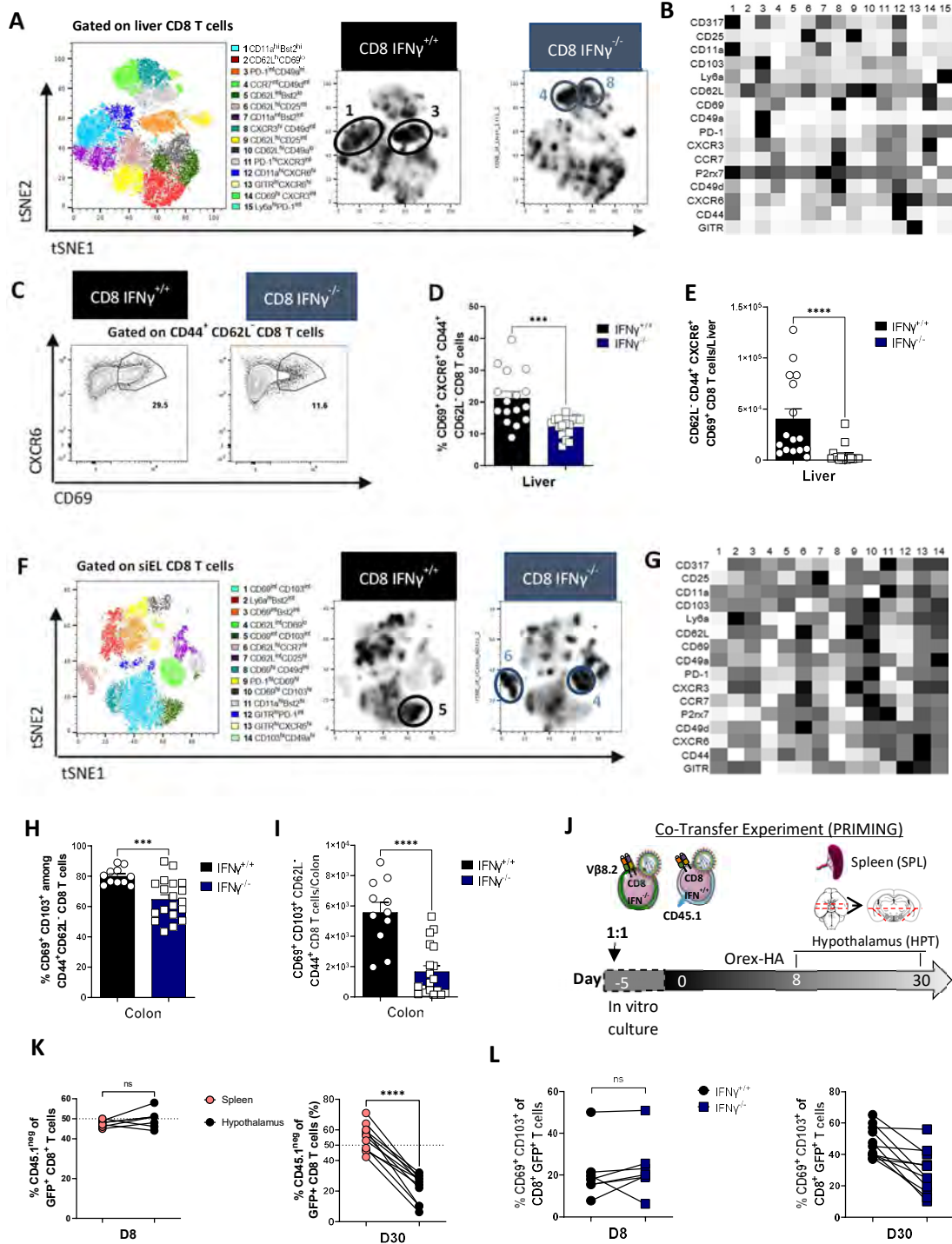
Besides this hypothesis, we also wondered if this cell-intrinsic effect comes from the lack of IFN $\gamma$  signaling during the priming phase in the *in vitro* culture. Therefore, we mixed IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> HA-specific CD8 T cells together from the beginning of the *in vitro* culture and co-transferred them as before in Orex-HA mice (**Supp. Fig 2.3J**). But again, the very same deficiency in the persistence and expression of T<sub>RM</sub> markers among IFN $\gamma$ <sup>-/-</sup> CD8 T cells was observed (**Supp. Fig 2.3K+L**).

In two other tissues, the liver and the colon, we performed high-dimensional flow cytometry to study the impact of IFN $\gamma$  deficiency on several T<sub>RM</sub> markers. Importantly, in the liver a CD8 T<sub>RM</sub> population expressing CD69 and CXCR6 was reduced in absolute and relative numbers in IFN $\gamma$ <sup>-/-</sup> compared to IFN $\gamma$ <sup>+/+</sup> mice (**Supp. Fig 2.3A-E**). Like the brain, a lower percentage and number of CD8 T<sub>RM</sub> cells expressing CD69 and CD103 were found in the colon of IFN $\gamma$ <sup>-/-</sup> compared to IFN $\gamma$ <sup>+/+</sup> mice (**Supp. Fig 2.3F-I**). These data indicate that IFN $\gamma$  deficiency impacts the persistence of CD8 T<sub>RM</sub> cells in several organs and that IFN $\gamma$  signaling might have a general role in the survival of CD8 T<sub>RM</sub> cells in non-lymphoid tissues.



**Figure 2.3 IFN $\gamma$  deficiency impairs CD8 T<sub>RM</sub> maintenance in the CNS (A)** Scheme of the experimental design: Orex-HA mice (CD45.1<sup>-</sup>) were adoptively transferred i.v. with HA-specific CD8 T cells (IFN $\gamma$ <sup>+/+</sup> or IFN $\gamma$ <sup>-/-</sup>) which were transduced with a GFP-expressing retrovirus during the *in vitro* activation phase/step. **(B)** Number of GFP<sup>+</sup> CD8 T cells (CD45.2<sup>+</sup>CD11b<sup>-</sup>TCR $\beta$ <sup>+</sup>CD8<sup>+</sup>GFP<sup>+</sup>) on day 8 in the spleen (*left*) and hypothalamus (*right*) of Orex-HA mice. Data from 8 to 10 mice per group in three independent experiments. **(C)** Number of GFP<sup>+</sup> CD8 T cells on day 30 in the spleen (*left*) and hypothalamus (*right*) of Orex-HA mice. Data from 7 to 9 mice per group in three independent experiments. **(D)** Representative FACS plots of CD69 and CD103 expression on transferred CD8<sup>+</sup> T cells (IFN $\gamma$ <sup>+/+</sup> or IFN $\gamma$ <sup>-/-</sup>) in the hypothalamus of Orex-HA mice, on days 8 and 30. **(E)** The proportion of GFP<sup>+</sup> CD8 T cells expressing CD69 and CD103 in the hypothalamus of Orex-HA mice at day 8 (*left*) or day 30 (*right*) from 7 to 10 mice per group in three independent experiments. **(F)** Scheme of the experimental design: Orex-HA-mice (CD45.1<sup>-</sup>) were adoptively transferred i.v. with both IFN $\gamma$ <sup>+/+</sup> (CD45.1<sup>+</sup>) and IFN $\gamma$ <sup>-/-</sup> (CD45.1<sup>-</sup>) HA-specific CD8 T cells that were transduced with a GFP-expressing retrovirus during the 5-day-long *in vitro* activation period. **(G)** Representative Histograms of CD45.1 expression among GFP<sup>+</sup> CD8 T cells (CD45.2<sup>+</sup>CD11b<sup>-</sup>TCR $\beta$ <sup>+</sup>CD8<sup>+</sup>GFP<sup>+</sup>) in the hypothalamus on day 8 (*top*) or day 30 (*bottom*) of Orex-HA mice. **(H)** Quantification of the ratio in % of IFN $\gamma$ <sup>-/-</sup> (CD45.1<sup>-</sup>) over IFN $\gamma$ <sup>+/+</sup> (CD45.1<sup>+</sup>) GFP<sup>+</sup> CD8 T cells in the spleen or hypothalamus on day 8 (*top*) or day 30 (*bottom*) of Orex-HA mice. Data are from 7 to 8 mice per timepoint of two independent experiments. **(I)** Proportion of CD69-expressing and CD103-expressing GFP<sup>+</sup> CD8 T cells in the IFN $\gamma$ <sup>-/-</sup> (CD45.1<sup>-</sup>) or IFN $\gamma$ <sup>+/+</sup> (CD45.1<sup>+</sup>) compartment in the hypothalamus on day 8 (*left*) or day 30 (*right*). Data are from 7 to 8 mice per timepoint of two independent experiments. **(J+K)** Representative histograms **(J)** and quantification **(K)** of the proportion of MHC-class II-expressing microglia (live CD45.2<sup>int</sup>CD11b<sup>int</sup>Ly-6c<sup>-</sup> cells) in the hypothalamus of Orex-HA transferred with either IFN $\gamma$ <sup>+/+</sup> or IFN $\gamma$ <sup>-/-</sup> CD8 T cells, or a mix of both cell types on day 8 (*left*) or day 30 (*right*). The data are from 7 to 10 mice per group and two to three independent experiments. Statistical analyses were performed using the Mann-Whitney test (B, C, E, F), Wilcoxon test (H, I), and one-way ANOVA with Tukey’s post hoc test (K). For all the panels, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

**Supplementary Figure 2.3 IFN $\gamma$  deficiency impairs CD8 T<sub>RM</sub> maintenance in the liver and the gut (A-B)** TSNE-graph with the cluster overlay and density plot of CD8<sup>+</sup> T cells from the liver of IFN $\gamma$ <sup>+/+</sup> or IFN $\gamma$ <sup>-/-</sup> mice. **(A)** and heatmap of the expression level of key markers **(B)** in the 15 identified clusters. **(C-E)** Representative FACS plots **(C)**, number **(D)**, and frequency of **(E)** CD69<sup>+</sup> CXCR6<sup>+</sup> CD8<sup>+</sup> CD44<sup>+</sup> CD62L<sup>-</sup> T cells in the liver from 16-17 mice per group of two independent experiments. **(F-H)** TSNE-graph with the cluster overlay and density plot of CD8<sup>+</sup> T cells from the colon of IFN $\gamma$ <sup>+/+</sup> or IFN $\gamma$ <sup>-/-</sup> mice. **(F)** and heatmap of the expression level of key markers **(G)** in the 14 identified clusters. **(H)** Number of CD44<sup>+</sup> CD62L<sup>-</sup> CD69<sup>+</sup> CD103<sup>+</sup> CD8 T cells between the two groups consisting of 11 to 19 mice of two independent experiments. **(I)** Proportion of CD44<sup>+</sup> CD62L<sup>-</sup> CD8 T cells expressing CD69 and CD103 in the colon (siEL). **(J)** Scheme showing the experimental design: Orex-HA-mice were adoptively transferred i.v. with HA-specific CD8<sup>+</sup> T cells (IFN $\gamma$ <sup>+/+</sup> and IFN $\gamma$ <sup>-/-</sup>) mixed from the beginning of the *in vitro* activation and transduced with a GFP-expressing retrovirus. **(K)** Quantification of the ratio of IFN $\gamma$ <sup>-/-</sup> (CD45.1<sup>-</sup>) over IFN $\gamma$ <sup>+/+</sup> (CD45.1<sup>+</sup>) GFP<sup>+</sup> CD8 T cells (TCR $\beta$ <sup>+</sup>CD8<sup>+</sup>GFP<sup>+</sup>) in the spleen or hypothalamus of day 8 (*left*) or day 30 (*right*) of Orex-HA mice. **(L)** Proportion of CD69 and CD103 expressing GFP<sup>+</sup> CD8 T cells in the IFN $\gamma$ <sup>-/-</sup> (CD45.1<sup>-</sup>) or IFN $\gamma$ <sup>+/+</sup> (CD45.1<sup>+</sup>) compartment of the hypothalamus on day 8 (*left*) or day 30 (*right*). Data are from 8 to 11 mice per timepoint of two independent experiments. Statistical analyses were performed using the Wilcoxon test (K, L) and Mann-Whitney test (D, E, H, I). For all the panels, \* p<0,05, \*\*p<0,01, \*\*\*p<0,001, \*\*\*\*p<0,0001.



#### 2.3.4 IFN $\gamma$ R1 deficiency abrogates CD8 T<sub>RM</sub> persistence in the CNS

As mentioned above, we hypothesized that IFN $\gamma$  signaling within CD8 T<sub>RM</sub> cells promotes their persistence in non-lymphoid tissues via an autocrine loop. In our previous study we identified by scRNA sequencing a cluster of HA-specific CD8 T cells on day 30 with an IFN-signaling gene signature. We also identified that hypothalamic CD8 T<sub>RM</sub> cells express high levels of IFN $\gamma$  (Frieser et al. 2022). Nevertheless, we do not know exactly whether this IFN-signaling gene signature was induced by type I or type II interferons, as both of them have similar gene targets mediated by STAT-1 phosphorylation and downstream signaling cascades. We first used this dataset to check out for the expression of IFN $\gamma$  receptors in HA-specific CD8 T cells from the spleen and the hypothalamus. As expected, IFN $\gamma$ R2 was not expressed, but IFN $\gamma$ R1 was expressed in CD8 T cells from both the spleen and hypothalamus (**Fig 2.4A**). By performing histological staining, we detected intranuclear pSTAT1 staining within CD8 T cells in the hypothalamus on day 30 in Orex-HA mice, suggesting active IFN $\gamma$  signaling within brain CD8 T<sub>RM</sub> cells (**Fig 2.4B**).

To study the impact of IFN $\gamma$  signaling on the CD8 T<sub>RM</sub> program, we aimed to knock out IFN $\gamma$ R1 on CD8 T cells. We chose the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) approach, as this system provides us with a pool of CD8 T cells that are sufficient and deficient for IFN $\gamma$ R1 because the gene editing is partial, leaving 10-20% of cells that are still expressing the receptor (**Fig 2.4C**). Due to the lack of a congenic marker, in this system the lack of expression of IFN $\gamma$ R1 helps us to trace the sgIFN $\gamma$ R1-transduced cells (**Fig 2.4D, E**). On day 8, no significant differences were observed in the proportion of IFN $\gamma$ R1 negative (IFN $\gamma$ R1<sup>neg</sup> – that had been transduced) between the spleen and the hypothalamus in the sgIFN $\gamma$ R1 group. Nevertheless, over time the IFN $\gamma$ R1<sup>neg</sup> compartment was overtaken by IFN $\gamma$ R1<sup>+</sup> expressing CD8 T cells, which were initially present at about 10% in the pool (**Fig 2.4F, G; top**). This phenomenon was not observed in the sgCTRL group, which indicates that this is not due to the gene-editing process (**Fig 2.4G; bottom**). Further characterization of the remaining hypothalamic IFN $\gamma$ R1<sup>neg</sup> CD8 T cells on day 30 revealed a reduced expression of classical T<sub>RM</sub> markers (CD69, CD103, CXCR3, and CXCR6) as well as markers associated with “exhaustion” (PD-1, Tigit, Slamf6) and IFN-regulated (Bst-2, Ly6a) T<sub>RM</sub> clusters (**Fig 2.4H-L**).

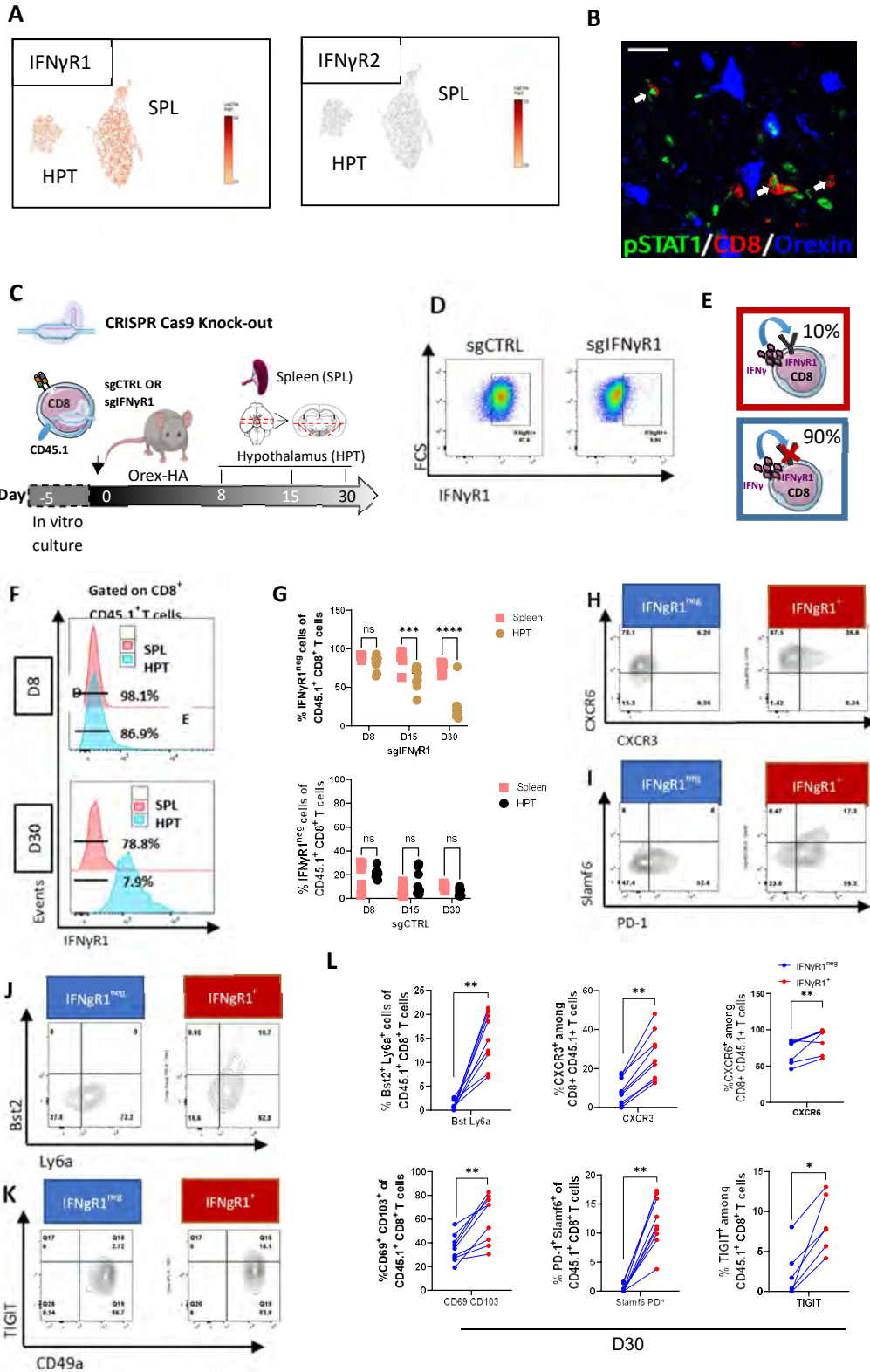
The cell number of transferred CD8 T cells did not differ much between the two groups in the spleen and the hypothalamus (**Supp. Fig 2.4A-C**). Only on day 30 were fewer sgIFN $\gamma$ R1 present in the spleen compared to the sgCTRL group (**Supp. Fig 2.4C**). On day 30, we

observed no difference in the frequency of  $T_{RM}$  markers between the sgIFN $\gamma$ R1 and sgCTRL groups, associated with a similar inflammation of the hypothalamus assessed by the MHC-II expression on microglia and presence of Treg cells (**Supp. Fig 2.4D-F**). These data indicate that the initially transferred IFN $\gamma$ R1<sup>+</sup> CD8 T cells over time almost completely overtake the IFN $\gamma$ R1<sup>neg</sup> CD8 T cells and sustain the inflamed state of the hypothalamus. This is associated with a reduced expression of CD8  $T_{RM}$  markers, which was already evident on day 15 (**Supp. Fig 2.4G**).

To control for possible off-target effects of the genome editing approach and to confirm the data with a different experimental strategy, we transduced CD8 T cells with a mife-based retroviral vector expressing shRNA (sh=short hairpin) that knock down the expression of either IFN $\gamma$ R1 or REN (luciferase not expressed in murine T cells). Importantly, the transduced CD8 T cells can be traced by the expression of the Thy1.1 reporter gene, which is included in the shRNA vector construct (**Fig 2.5A**). It is worthy of note, contrasting with the CRISPR-Cas9 approach, that the IFN $\gamma$  signaling is only partially impaired as the receptor is still expressed, albeit at lower levels than the control.

We first confirmed that the expression of the IFN $\gamma$ R1 is downregulated on day 8 and day 30 in the shIFN $\gamma$ R1 compared to the shREN group both in the spleen and the hypothalamus (**Fig 2.5B-D**). Next, we studied the frequency and number of transferred CD8 T cells expressing Thy1.1 in the spleen and the hypothalamus over time. In the spleen, the percentage (**Fig 2.5E, F; left**) and number (**Fig 2.5G, H; left**) of Thy1.1<sup>+</sup> CD8 T cells did not differ between the shIFN $\gamma$ R1 and the shREN groups at the two points in time. In contrast, in the hypothalamus, while similar frequencies and numbers of Thy1.1<sup>+</sup> CD8 T cells were found in both groups on day 8, the frequency and the absolute number of Thy1.1<sup>+</sup> CD8 T cells were strikingly reduced in the shIFN $\gamma$ R1 group on day 30 (**Fig 2.5E-H; right**).

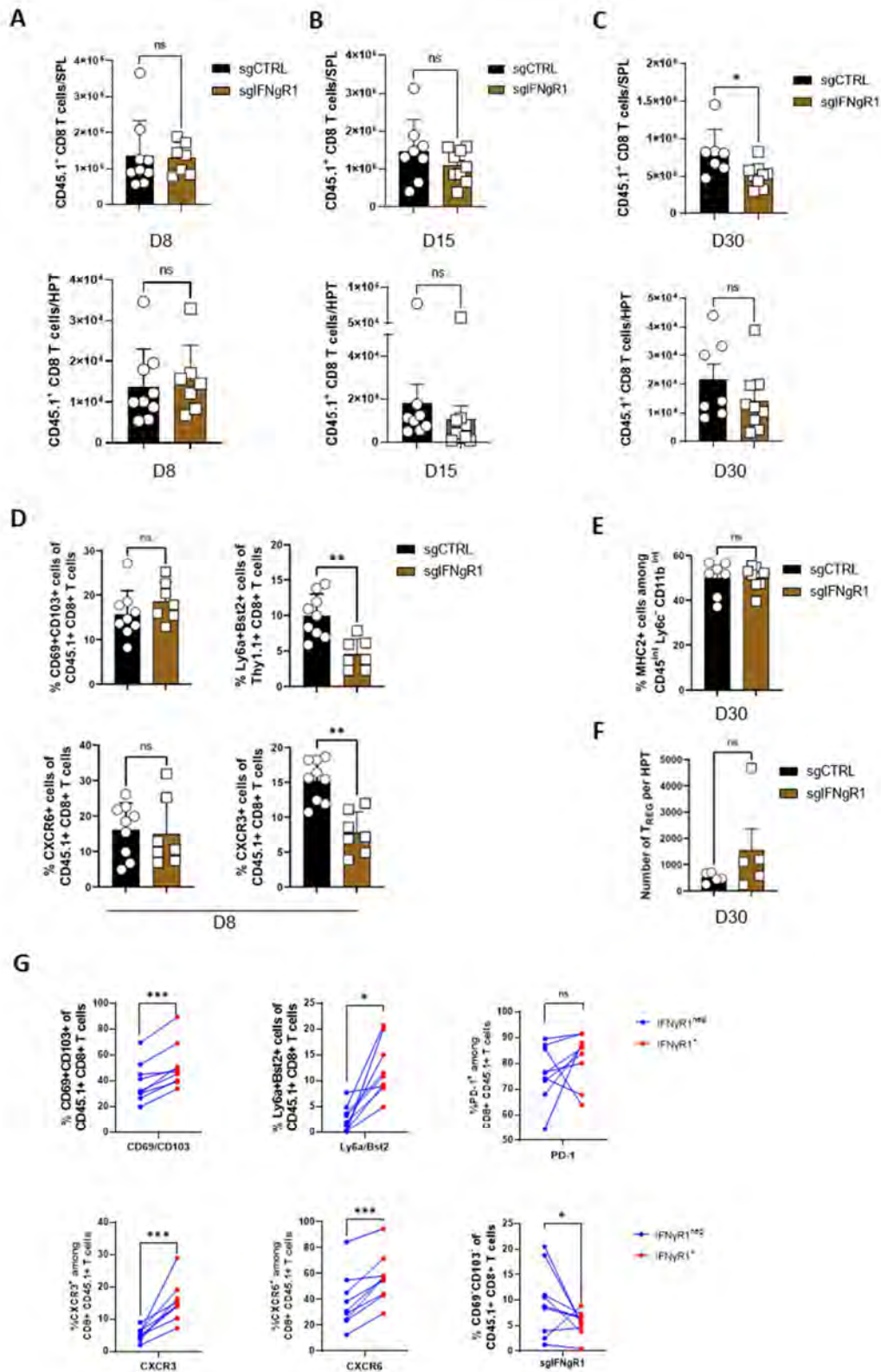
Regarding the phenotype, on day 8 transferred Thy1.1<sup>+</sup> CD8 T cells expressed lower levels of Bst-2 and Ly6a (markers for IFN-signaling identified in the scRNA sequencing), but similar levels of CD69 and CD103 (**Fig 2.5I**). On day 30, the remaining Thy1.1<sup>+</sup> CD8 T cells of the shIFN $\gamma$ R1 group expressed lower proportions of Bst-2, Ly6a, the chemokine receptor CXCR6 and molecules associated with exhaustion (Tim-3, Tigit) compared to shREN transduced CD8 T cells (**Fig 2.5J**). In sum, both impaired and absent IFN $\gamma$ R1 mediated signaling strongly abrogates the survival of CD8  $T_{RM}$  cells in non-lymphoid tissues. Everything considered, IFN $\gamma$  signaling is important for the pathogenicity of CD8 T cells in this CNS autoimmune setting.

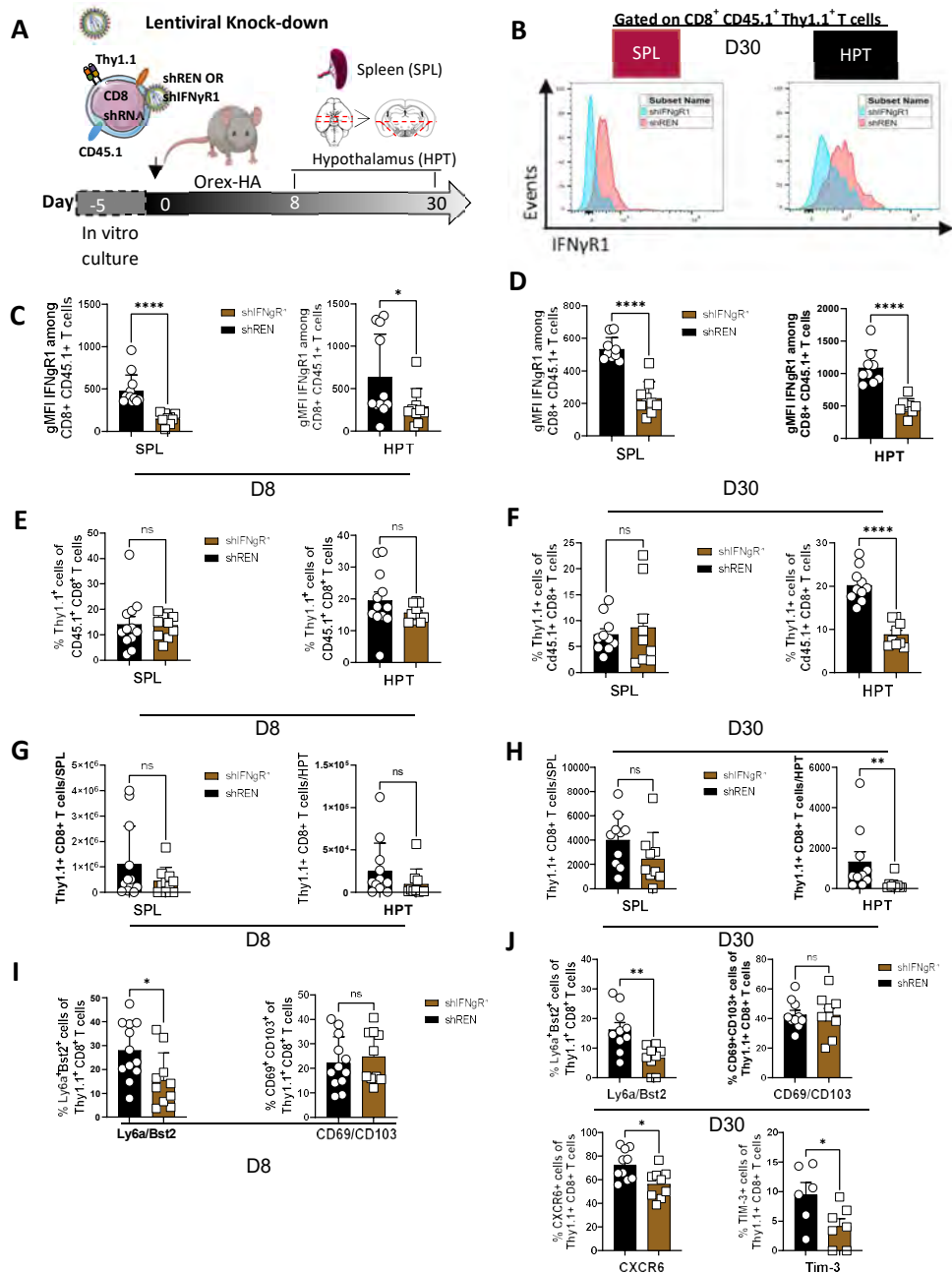




**Figure 2.4 IFN $\gamma$ R1 deficiency abrogates CD8 T<sub>RM</sub> persistence in the CNS (A)** UMAP representation of IFN $\gamma$ R1 or IFN $\gamma$ R2 expressing cells of scRNA-sequencing data of splenic and hypothalamic transferred CD8<sup>+</sup> T cells from Orex-HA mice, on day 30. **(B)** Triple staining for pSTAT1 (green), CD8 (red), and orexin (blue) in the hypothalamus of Orex-HA mice, on day 30. The inset shows a pSTAT1 nuclear staining in CD8<sup>+</sup> cells among orexin-expressing neurons. Scale bar: 25  $\mu$ m. **(C)** Scheme showing the experimental design: Orex-HA-mice were adoptively transferred i.v. with HA-specific CD8 T cells transfected with sgCas9 vectors (sgIFN $\gamma$ R1 or sgREN) from the beginning of the *in vitro* culture. **(D)** Representative FACS plot staining of IFN $\gamma$ R1 among gene-edited CD8<sup>+</sup> T cells after *in vitro* culture. **(E)** Explanatory model to explain that the adoptive transfer consists of about 10% that still express IFN $\gamma$ R1. **(F)** Representative histogram of IFN $\gamma$ R1 expression cells of hypothalamic CD45.1<sup>+</sup> CD8 T cells on day 8 (*top*) and day 30 (*bottom*). **(G)** Quantification of IFN $\gamma$ R1 negative cells in the hypothalamus and spleen from the sgIFN $\gamma$ R1 (*top*) and sgCTRL (*bottom*) group at different points in time. **(H-K)** Representative FACS plots of the proportion of CXCR6 and CXCR3 **(I)**, Slamf6 and PD-1 **(J)**, of Bst-2 and Ly6a **(K)**, as well as TIGIT and CD49a **(L)** expressing hypothalamic CD45.1<sup>+</sup> CD8 T cells on day 30 in the sgIFN $\gamma$ R1 group, stratified according to their expression or lack of IFN $\gamma$ R1. **(M)** Quantification of CXCR6, CXCR3, Slamf6/PD-1, Bst-2/Ly6a, Tigit, as well as CD69/CD103 expression. Data are from 9 mice in two independent experiments. Statistical analyses were performed using Two-way ANOVA with Sidak’s post hoc test (G, H) and Wilcoxon test (M). For all the panels, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

**Supplementary Figure 2.4 IFN $\gamma$ R1 deficiency abrogates CD8 T<sub>RM</sub> persistence in the CNS (A-C)** Number of CD45.1<sup>+</sup> CD8 T cells in the spleen (*top*) or hypothalamus (*bottom*) of Orex-HA mice 8 days after transfer of sgIFN $\gamma$ R1 (brown) or sgCTRL (black) gene-edited HA-specific CD8 T cells. Data from day 8 are from 7 to 9 mice per group from two independent experiments. Data from day 15 containing 5 to 6 mice per group of one experiment **(B)**. Data from day 30 containing 7 to 9 mice per group of two independent experiments **(C)**. **(D)** Proportion of CXCR6, CXCR3, Bst-2/Ly6a as well as CD69/CD103 expression cells of hypothalamic CD45.1<sup>+</sup> CD8 T cells in the sgIFN $\gamma$ R1 or sgCTRL group, on day 8. **(E)** Proportion of MHC-II-expressing microglia of the sgIFN $\gamma$ R1 or sgCTRL group, on day 30. **(F)** Numbers of hypothalamic Treg cells (TCR $\beta$ <sup>+</sup> CD4<sup>+</sup> FoxP3<sup>+</sup>) in the sgIFN $\gamma$ R1 or sgCTRL group, on day 30. Data are from one experiment including 5 to 6 mice. **(G)** Proportion of CXCR6, CXCR3, PD-1, Bst-2/Ly6a, CD49a as well as CD69 and CD103 expression of hypothalamic CD45.1<sup>+</sup> CD8 T cells in the sgIFN $\gamma$ R1 group on day 30, which express or lack IFN $\gamma$ R1. Statistical analyses were performed using the Mann-Whitney test (A-F) and Wilcoxon test (G). For all the panels, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.





**Figure 2.5 IFN $\gamma$ R1 mediated signaling promotes T<sub>RM</sub> maintenance in the CNS (A)** Scheme of the experimental design: Orex-HA mice (CD45.1<sup>+</sup>) were adoptively transferred i.v. with CD45.1<sup>+</sup> HA-specific CD8 T cells transduced with retroviral shRNA vectors (shIFN $\gamma$ R1 or shREN) during the *in vitro* activation step. Both retroviral shRNA vectors induce the expression of the Thy1.1 marker. **(B)** Representative histogram of IFN $\gamma$ R1 expression on Thy1.1<sup>+</sup> CD45.1<sup>+</sup> CD8 T cells transduced with shIFN $\gamma$ R1 or shREN 8 in the spleen (*left*) or hypothalamus (*right*) of Orex-HA mice, on day 30. **(C-D)** Quantification of the geometric mean fluorescence intensity of IFN $\gamma$ R1 in the two groups among Thy1.1<sup>+</sup> CD45.1<sup>+</sup> CD8 T cells in the spleen (*left*) or hypothalamus (*right*) of Orex-HA mice, on day 8 **(C)** and day 30 **(D)**. **(E-G)** Proportion **(E)** or number **(F)** of Thy1.1 expressing cells in the spleen (*left*) or hypothalamus (*right*) of Orex-HA mice receiving CD45.1<sup>+</sup> CD8 T cells transduced with shIFN $\gamma$ R1 (brown) or shREN (black), on day 8. Data from 11 to 12 mice per group from three independent experiments. Proportion **(G)** or number **(H)** of Thy1.1 expressing cells in the above-mentioned organs, on day 30. Data from 9 to 10 mice per group from two independent experiments. **(I)** Proportion of CD69 and CD103 (*left*) or Bst-2 and Ly6a (*right*) expressing Thy1.1<sup>+</sup> CD45.1<sup>+</sup> CD8 T cells in the hypothalamus transduced with shIFN $\gamma$ R1 or shREN, on day 8. Data are from 11 to 12 mice per group from three independent experiments. **(J)** Percentage of Bst-2 and Ly6a, CXCR6, CD69, and CD103 or TIM-3 expressing Thy1.1<sup>+</sup> CD45.1<sup>+</sup> CD8 T cells transduced with shIFN $\gamma$ R1 or shREN, in the hypothalamus of Orex-HA mice on day 30. Except for Tim-3 (6 to 7 mice from one experiment) data are from 9 to 10 mice per group of two independent experiments. Statistical analyses were performed using the Mann-Whitney test (C-J). For all the panels, \* p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

## 3 Discussion

### 3.1 CD4 T cells and CD8 T cells display pathogenic characters in autoimmune mouse models of Narcolepsy type I

In this work, we developed a new autoimmune mouse model for Narcolepsy Type I based on the vaccination with Pandemrix®. The aim was to study the mechanisms by which the vaccination induced loss of orexin neurons – a key feature of NT1. In this study, Pandemrix® vaccination itself was not sufficient to trigger orexin loss in Orex-HA mice but required an enrichment in HA-specific CD4 and CD8 T cells in the mouse repertoire to trigger neuronal loss. In response to the vaccine, both HA-specific CD4 and CD8 T cells produced high levels of IFN $\gamma$  and TNF, and CD4 T cells also produced IL-17. The injection of HA-specific CD8 T cells alone was not sufficient for T cells to cross the BBB and induce neuronal loss. In contrast, the transfer of HA-specific CD4 T cells alone was sufficient to induce hypothalamic inflammation and neuronal loss. Nevertheless, this work provided evidence that the early depletion of CD8 T cells abrogated the neuronal loss, pointing towards an important role of this T cell subset in the destruction of orexinergic neurons. Once in the hypothalamus, both the endogenous compartment of CD4 and CD8 T cells shared similar gene expression profiles with the respective transferred T cells. It is noteworthy that CD8 T cells in the hypothalamus showed a CD8 T<sub>RM</sub> gene signature. Flow Cytometry, sequencing, and histological data coupled with CD4 and CD8 T cell depletion strategies pointed convergently towards an important role of CD8 T<sub>RM</sub> cells to drive chronic tissue damage to orexin neurons.

Several studies attempted to identify an immune signature in the blood of patients developing NT1 following vaccination with Pandemrix®. Nevertheless, most of those studies did not find differences between vaccinated and non-vaccinated NT1 cases regarding cytokines, change of the CD4 or CD8 T cell memory subsets, or expanded T cell clones (Hartmann et al. 2016, Beltran et al. 2019). A possible explanation is that the blood samples were taken several months or even years later after the actual onset of NT1 (and Pandemrix® vaccination), which might have diluted possible changes in the T cell compartments or changes in cytokine levels. Another explanation is that other NT1 patients might have developed the disease in response to viral or streptococcal infection and therefore the change in T cell compartments is quite similar to vaccinated individuals (Longstreth et al. 2009, Han et al. 2011). In any event, current data indicate that Pandemrix® vaccine-associated NT1 and idiopathic NT1 are similar in terms of genetic susceptibility and immunological profile. In our study, we highlighted the fact that CD4

and CD8 T cells which responded to the vaccine produced IFN $\gamma$ , TNF, and IL-17 (only CD4 T cells), which indicates that Pandemrix elicits a mixed type I and III immune response.

Furthermore, the association of Pandemrix<sup>®</sup> vaccination and NT1 onset was hypothesized to involve molecular mimicry: Substances from the viral particles in the vaccine might have elicited cross-reactivity with an autoantigen expressed in orexin neurons. Molecular mimicry was demonstrated in several neurological autoimmune diseases: Certain lipo-oligosaccharides from *Campylobacter jejuni* induce the production of antibodies directed against nerve gangliosides resulting in Guillain-Barré syndrome (Godschalk et al. 2004; Laman et al. 2022). Furthermore, in patients with Sydenham Chorea, a movement disorder occurring after group A *Streptococcus* infection, cross-reactive autoantibodies against mammalian lysoganglioside and the streptococcal N-acetyl-beta-D-glucosamine (GlcNAc) were detected (Kirvan et al. 2003). In MS, cross-reactive autoantibodies were detected between an EBV epitope and the CNS protein GliaCAM (Lanz et al. 2022). Besides, as already discussed for PCD cases, the expression of a neuronal antigen by a tumor can induce the generation of autoantibodies and autoreactive T cells directed towards antigens expressed in CNS neurons.

One study reported the detection of autoantibodies directed against orexin receptor 2 in 85% of Pandemrix<sup>®</sup> vaccinated NT1 cases that were cross-reacting with nucleoprotein A present in the vaccine components (Ahmed et al., 2015). Nevertheless, orexin receptor 2 is not expressed by orexin neurons (Vasalli et al. 2015) and, therefore, these autoantibodies cannot explain the specific loss of orexin neurons. It might rather be the consequence than the cause of NT1 onset. Further studies reported that T cell clones recognizing the C-amidated orexin peptide Orexin<sub>54-66</sub> share similar TCR clonotypes with T cell clones that recognize the Influenza A epitopes pHA<sub>273-287</sub> (Luo et al. 2018). Nevertheless, a follow-up study cloned 709 identified TCRs into Jurkat 76 T cells and found that functional TCRs did not recognize orexin or Influenza A epitopes (Luo et al. 2022). This study, as well as a previous study by Latorre et al. 2018, does not provide evidence in favor of the hypothesis that NT1 onset is induced by molecular mimicry.

Our goal was to design a reductionist mouse model to assess whether and how vaccination with Pandemrix<sup>®</sup> could induce a narcolepsy-like phenotype. Thus, we created a system that reflects the activation in the periphery of cross-reactive T cells, which later on cause damage in the CNS to orexin neurons. Beyond molecular mimicry, other mechanisms can be thought of such as bystander activation: T cells were shown to be enabled to cross the BBB upon strong infections (Klein et al. 2017). The adjuvant AS03 mainly activates the innate immune systems

to stimulate antigen uptake and presentation (Garcon et al. 2012). Besides, AS03 also induces the expression of other cytokines and chemokines such as IFN $\gamma$ , which could alter the permissiveness of the BBB to allow lymphocytes to transmigrate to the CNS (Morel et al. 2011). One could think that a strong adjuvant such as AS03 elicited certain autoreactive T cells to enter the hypothalamus and induce the loss of orexinergic neurons. In our experiments, we observed inconstant and modest T-cell infiltration in the hypothalamus by the injection of solely the adjuvant AS03. However, this only happened when the repertoire was enriched for both naïve, HA-specific CD4 and CD8 T cells. Taken together, upon Pandemrix<sup>®</sup> vaccination orexin neuron loss was solely observed, when autoreactive CD4 and CD8 T cells were present in the mouse T cell repertoire. Sometimes hypothalamic inflammation was seen after injection of the adjuvant AS03 only. This speaks in favor of AS03 as potent adjuvant, that could possibly trigger the breakdown of the BBB.

Importantly, the enrichment in naïve, HA-specific CD4 T cells was sufficient to initiate the neuronal loss. Those observations are interesting given the immune signature of NT1 patients: Mainly orexin-specific, autoreactive CD4, but more rarely CD8 T cells are found in the blood of NT1 patients (Lattore et al 2018; Luo et al. 2018; Jiang et al. 2019). Interestingly, AS03 was shown to elicit a potent polyfunctional CD4 T cell response (Moris et al. 2011). Furthermore, the association of the increase in NT1 relative incidence was solely observed in carriers of the HLA-DQB:06\*02 allele of the MHC-II complex (Szakács et al. 2013). This could lead to the hypothesis that the genetic predisposition favored the development of autoreactive CD4 T cells in NT1 patients. Other environmental factors such as preceding influenza (Han et al. 2011) or streptococcal infections (Aran et al. 2009) could have further enriched the T cell repertoire with autoreactive CD4 and/or CD8 T cells. Everything considered, Pandemrix<sup>®</sup> vaccination using the very potent AS03 adjuvant might have been the final trigger to induce hypothalamic infiltration and the onset of NT1.

It is noteworthy that our data demonstrated that autoreactive CD4 T cells were unable to induce neuronal loss in the absence of CD8 T cells. This suggests that although CD4 T cells help CD8 T cells to cross the BBB, the latter are the executors of neuronal destruction. This is supported by the high expression of *GRZB*, *FASL*, *GM-CSF*, *IFNG*, and *TNF* by both endogenous and transferred CD8 T cells revealed by our RNA sequencing dataset. Furthermore, flow-cytometry data confirmed the expression of GM-CSF, IFN $\gamma$ , and TNF of hypothalamic CD8 T cells in response to HA peptides. Besides, CD8 T cells were found in close association with orexin neurons, polarizing Granzyme B towards them. Collectively, the data speak in favor of the

direct killing of orexin neurons by the transfer of cytotoxic molecules from cytotoxic CD8 T cells to the neurons.

CD8 T cells could induce direct neuronal lysis in several autoimmune diseases of the CNS: In RE patients, CD8 T cells were found polarizing Granzyme B towards CNS neurons (Schneider-Hohendorf et al. 2016, Wang et al. 2022), which were also stained positive for Caspase-3 (Bien et al. 2002). A similar mechanism can be detected in post-mortem samples of PCD patients, demonstrating CD8 T cells polarizing Granzyme B towards MAP2 neurons (Bien et al. 2012). Furthermore, brain and CSF CD8 T cells from MS patients exhibit high expression of Granzyme B and other cytotoxic molecules as revealed by histology (Machado Santos et al. 2018), flow cytometry (Hsiao et al. 2021) and scRNA sequencing (Beltran et al 2019; Pappalardo et al. 2020). In the brain parenchyma of progressive MS patients, 76% of CD8 T cells with a T<sub>RM</sub> phenotype expressed Granzyme B (Vincenti et al. 2022)

In line with this, our scRNA-sequencing dataset in the adoptive transfer model of effector HA-specific CD8 T cells revealed that the largest cluster of hypothalamic CD8 T cells expressed high levels of several cytotoxic molecules including Granzyme A and B as well as Perforin. Interestingly, the sequencing of activated CD4 or CD8 T cells in the blood of NT1 patients also showed higher expression of Granzyme B and Perforin compared to controls (Vuorela et al. 2021, Luo et al. 2022) and a loss-of-function mutation in the Perforin gene is a protective allele for NT1 development (Ollila et al. 2018). Convergenly, those data suggest that cytotoxic molecules from T cells might play a role in orexin neuron destruction. In our mouse model, we could test the impact of the genetic depletion of Perforin and Granzyme B on neuronal loss, in order to assess whether those mechanisms are required for neuronal loss.

We further found an interesting cluster of CD8 T<sub>RM</sub> cells that expressed exhaustion-related genes (Tox, PD-1, and Tim-3). Nevertheless, the proliferation marker Ki-67 suggests that despite the expression of inhibitory molecules, those CD8 T<sub>RM</sub> cells were well responsive to antigen re-stimulation. Another CNS autoimmune model highlighted that brain CD8 T<sub>RM</sub> cells expressed Tox and the genetic depletion of Tox resulted in the reduction of CD8 T<sub>RM</sub> cells in the brain and consistently reduced tissue damage (Page et al. 2018 and 2021). Interestingly, this was observed in the context of autoimmunity, but not a viral infection of the CNS. It might be that the expression of inhibitory molecules hinders CD8 T<sub>RM</sub> cells from being over-activated and undergoing activation-induced cell death due to the constant presence of the antigen in autoimmune settings. Besides, clonally expanded CD8 T cells express several exhaustion-related genes in the CSF of MS patients (Pappalardo et al. 2020).

In this Ph.D. Thesis we provided further evidence that autoreactive effector CD8 T cells differentiate into a tissue-resident population in the hypothalamus, using a mouse model of autoimmunity against orexin neurons. A stable pool of CD69<sup>+</sup> CD103<sup>+</sup> CD8 T<sub>RM</sub> cells populated the hypothalamus one week after adoptive transfer and resided there for at least three months. This CD8 T cell population was resistant to systemic depletion and was able to cause the loss of orexin neurons independently from newly incoming CD8 T cells from the periphery. We observed that CD8 T<sub>RM</sub> cells drive chronic CNS damage independently of CD8 T cells from the periphery. These data suggest that CD8 T<sub>RM</sub> cells can drive chronic damage to orexin neurons.

In recent years several studies identified tissue-resident T cells in various diseases of the CNS, especially in the context of viral infections (Wakim et al. 2010 and 2012, Prasad et al. 2015 and 2017, Steinebach et al. 2016 and 2019, Baxter et al. 2020 among other publications). Nevertheless, the understanding of the role of T<sub>RM</sub> cells in the pathological context of autoimmune diseases of the CNS is just emerging. The scientific community still needs a deeper understanding of how T<sub>RM</sub> cells contribute to chronic CNS damage.

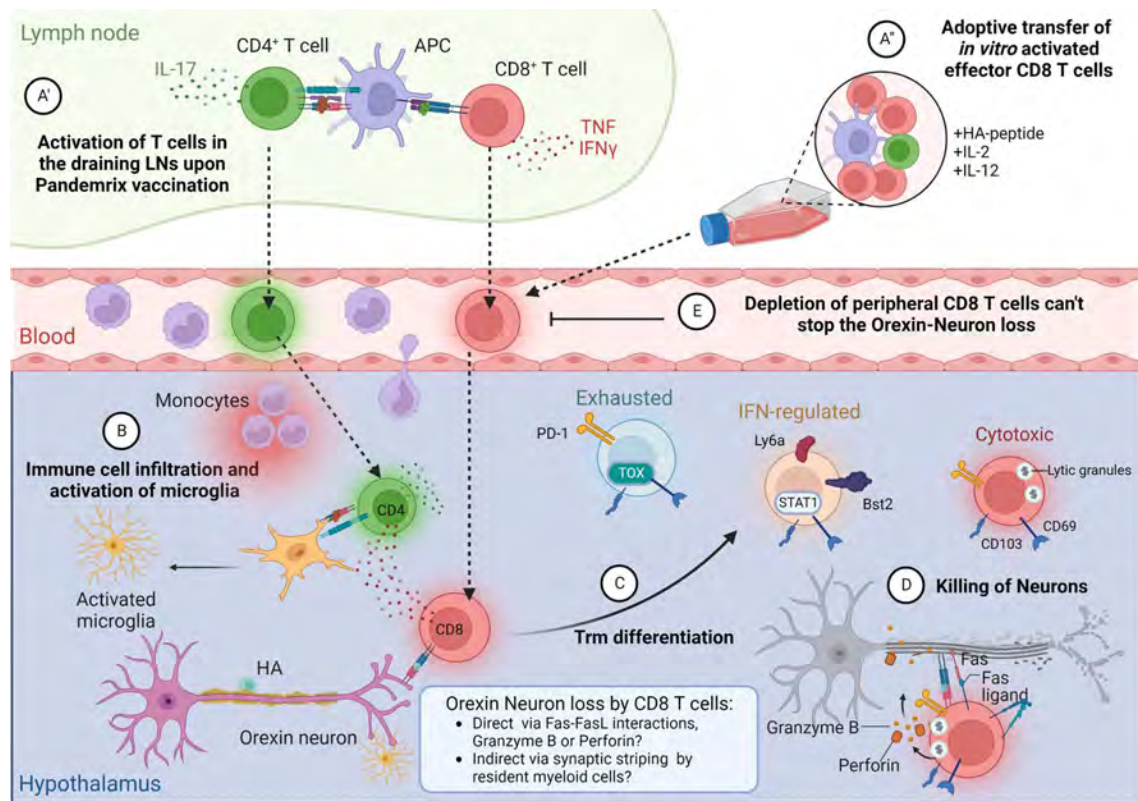
In the context of autoimmunity, T<sub>RM</sub> cells are discussed to be key drivers of tissue damage and might represent an interesting therapeutic target. In MS patients, the administration of fingolimod (targeting circulating but not resident T cells) appears to be mostly inefficient in the secondary progressive disease phase. Interestingly, the administration of depleting CD20 monoclonal antibodies is one of the most efficient therapies for the treatment of relapsing MS targeting mainly B cells (Naegelin et al. 2019). Nevertheless, although CD20 is normally expressed in B cells, a recent study highlighted that CD8 T<sub>RM</sub> cells in active MS lesions express intermediary levels of CD20 and could therefore be also targeted by anti-CD20 mAb treatment (Smolders et al. 2020, Hsiao et al. 2021). However, specific therapeutics targeting T<sub>RM</sub> cells still need to be discovered.

In active lesions of MS patients, a high number of CD8 T cells were detected expressing CD69, CD103, CXCR6, and cytotoxicity-related molecules compared to normal-appearing white matter (Fransen et al. 2020). Single-cell RNA sequencing of T cells in the CSF of monozygotic twins with MS compared to their healthy twin highlighted that expanded CD8 T cell clones, showed a tissue-resident gene expression program, involving upregulation of tissue adhesion molecules and down-regulation of genes involved in tissue egress (Beltran et al. 2019). This was confirmed in subsequent studies showing the presence of clonally expanded CD4 and CD8 T cells in the CSF expressing several tissue-residency-related genes (Papalardo et al. 2020).



Similarly, in RE, CD4 and CD8 T cells expressing residency markers were described up to 8 years after disease onset (Owens et al. 2016). In our samples, we studied for the first time the phenotype of CD8 T cells in the brain parenchyma of paraneoplastic neurological disease patients and found a high density of CD8 T cells expressing tissue residency markers. Furthermore, those cells expressed exhaustion-related proteins (TOX, PD-1) and Granzyme B. It would be very interesting to have access to sequencing data of CD8 T cells from the CSF of NT1 patients to see if expanded CD8 T cell clones have features of CD8 T<sub>RM</sub> cells. This might then indicate that CD8 T<sub>RM</sub> cells can be involved in the pathophysiology of NT1.

Everything considered, the following illustration summarizes key findings of pathogenic mechanisms at work in the loss of orexin neurons (Fig 3.1).



**Figure 3.1 Both CD4 T cells and CD8 T cells display pathogenic characters in two autoimmune models of Narcolepsy type I** (A) In A', the vaccine model is depicted in a simplistic way, where the vaccination with Pandemrix® leads to antigen presentation of HA-peptides to CD4 T cells and CD8 T cells. In A'' the adoptive transfer model is described, where CD8 T cells are activated in the presence of irradiated Splenocytes, the HA<sub>512-520</sub>-peptide, IL-2, and IL-12 during five days of *in vitro* culture. (B) Activated CD4 and CD8 T cells then migrate to the hypothalamus and create local inflammation measured by the activation of microglia and the recruitment of other immune cells such as inflammatory monocytes. (C) We provided evidence that CD8 T cells differentiate into T<sub>RM</sub> cells in an antigen-specific manner and form a heterogeneous pool with three distinct clusters demonstrated by our scRNA sequencing data. (D) The exact mechanisms involved in orexin neuron loss remain elusive, but it might happen via polarization of lytic granules containing Granzyme B and Perforin towards their target.

### 3.2 CD4 T cells provide helper signals for the effective priming of CD8 T cells and their persistence in the hypothalamus

In the vaccination model, we observed that the transfer of HA-specific CD8 T cells alone was unable to induce hypothalamic inflammation and neuronal loss. In contrast, CD8 T cells in the presence of HA-specific CD4 T cells entered the hypothalamus and induced neuronal loss.

This can be explained by the importance of help given by CD4 T cells to CD8 T cells. In our experiments we demonstrated that transferred HA-specific CD8 T cells proliferated in the same way as CD8 T cells co-transferred with HA-specific CD4 T cells in response to the vaccine. Furthermore, they produced similar levels of IFN $\gamma$  and TNF. Nevertheless, the ‘unhelped’ CD8 T cells could not enter the hypothalamus. It was shown that CD4 T cells help CD8 T cells to acquire the expression of chemokine receptors and metalloproteases (Ahrends et al. 2017), which enables them to enter non-lymphoid tissues such as the brain (Sitati et al. 2007). Furthermore, CD4 T cells help induce the expression of cytotoxic molecules in CD8 T cells such as Granzyme B (Provine et al. 2016; Ahrends et al. 2017). In our experiments, among ‘unhelped’ CD8 T cells on day 5 after Pandemrix<sup>®</sup> vaccination we observed a reduced Granzyme B expression. Nevertheless, we did not look at chemokine receptors and integrins such as CD11a, CD49d, or CXCR4 which are important molecules involved in the migration to the CNS.

We further highlighted that the persistence of CD8 T cells required the help of *in situ* lodged CD4 T cells. Those hypothalamic CD4 T cells displayed a T<sub>FH</sub> phenotype. The absence of CD4 T cells resulted in a reduced number of CD8 T<sub>RM</sub> cells in the hypothalamus, provided CD8 T cells were concurrently depleted in the periphery.

The need for CD4 T cell help for CD8 T<sub>RM</sub> cell survival in the tissue was first identified in brain infections with Poliovirus (Mockus et al. 2018, Ren et al. 2020) and lung infection with influenza virus (Son et al. 2021). Ren et al. showed that the combination of CD4 T cells and CD8 T cell depletion in the periphery was required to reduce the number of CD8 T cells in the lung, which resulted in reduced viral control. Similarly, in our model, the double depletion of CD4 T cells and CD8 T cells reduced the number of CD8 T<sub>RM</sub> cells in the hypothalamus and subsequently partially restored the number of orexin neurons. When we depleted only CD4 T cells, the number of CD8 T<sub>RM</sub> cells was not impacted.

A possible explanation for this is that CD8 T cells from the periphery can replenish the emptied niche in the tissue. In favor of this hypothesis, the phenotype of newly incoming CD8 T cells was different, with reduced expression of Granzyme B, Tox, and PD-1. Therefore, this Ph.D. thesis provided evidence that helper signals from *in situ* lodged CD4 T cells are important beyond survival in the functional properties of CD8 T<sub>RM</sub> cells.

The study of Son et al. highlighted that those CD4 T cells have a phenotype of resident T<sub>FH</sub> cells. We also found hypothalamic CD4 T cells to express CD69, CXCR6, ICOS, and PD-1 as markers of residency and T<sub>FH</sub>-phenotype. Due to the lack of convincing IL-21 staining and the lack of adequate reporter mice, we could not test if those CD4 T cells secrete IL-21 and sustain the persistence of CD8 T cells by this mechanism. Furthermore, CD4 T cells could also have secreted IFN $\gamma$  and sustained CD8 T<sub>RM</sub> cells by this mechanism, as will be further detailed below. We performed PMA/Ionomycin stimulation of hypothalamic CD4 T cells and found more than 80% to secrete IFN $\gamma$  in this auto-immune setting.

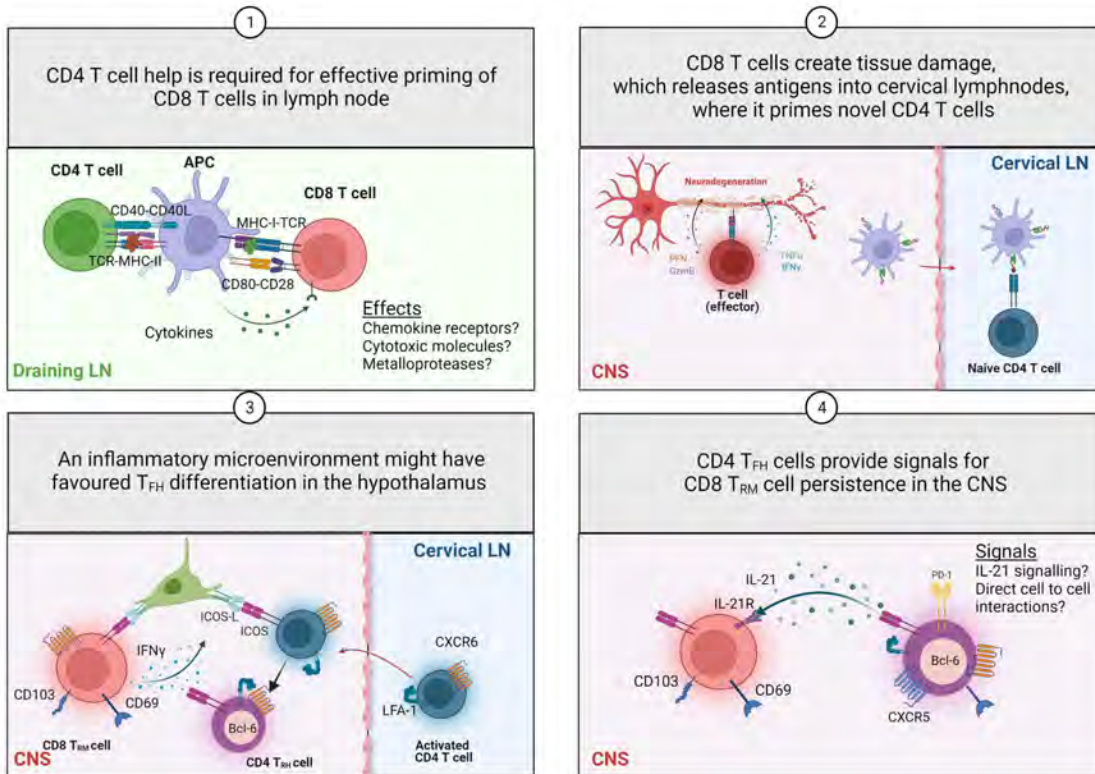
It is noteworthy that when we depleted CD4 T cells later, starting on day 8 post transfer of autoreactive CD8 T cells, the numbers of CD8 T<sub>RM</sub> cells as well as CD69, CXCR6, ICOS, and PD-1 expressing CD4 T cells in the hypothalamus were not impacted. This further strengthens the idea that those T<sub>FH</sub>-like cells are resident. Taken together, there might be a positive feedback loop, wherein the CD8 T cell-mediated tissue damage induces the recruitment of CD4 T cells, which themselves become resident and sustain the tissue damage, by supporting the survival of autoreactive CD8 T cells leading to further tissue injury.

One question in this project was to evaluate how CD4 T<sub>FH</sub> cells are established in the hypothalamus as, in the CD8 T cell transfer paradigm, neither viral infection nor vaccination could have triggered their activation in the periphery. These cells could have been recruited to the hypothalamus by an antigen-independent mechanism due to the local immune activation. Given the transfer of about 3 million effector T cells producing high levels of IFN $\gamma$  and TNF, the strong inflammation in the hypothalamus could have induced the breakdown of the BBB. A study highlighted that IFN $\gamma$  signaling in brain endothelial cells leads to cytoskeletal changes *in vitro*, which might explain the opening of the BBB (Bonney et al. 2019). Furthermore, the chemokine gradients produced by neurons under CD8 T cell attack might have directed the incoming CD4 T cells to the place of inflammation. This idea would be supported by the fact that orexin neurons on day 8 show gene expression of CXCL10 and CXCL16. Interestingly, a high proportion of hypothalamic CD4 T cells on day 30 expressed the chemokine markers CXCR3 and CXCR6, which could have guided those to the site of tissue damage.

Besides,  $\text{IFN}\gamma$  produced by CD8  $T_{\text{RM}}$  cells might have even induced the differentiation of CD4 T cells into  $T_{\text{FH}}$  cells. It was indeed shown that excess of  $\text{IFN}\gamma$  in the San Roque lupus model leads to the accumulation of  $T_{\text{FH}}$  cells. Mechanistically it was shown that  $\text{IFN}\gamma$  induces Bcl-6 expression (Lee et al. 2012). Furthermore, ICOS signaling is not only important for CD8  $T_{\text{RM}}$  formation, but also for  $T_{\text{FH}}$  maintenance in the tissue by upholding Bcl-6 expression (Choi et al. 2011). It is conceivable that CD8  $T_{\text{RM}}$  cells create a specific microenvironment which favors the establishment of  $T_{\text{FH}}$  cells in the tissue. Data from our team suggest that  $\text{IFN}\gamma$  upregulates ICOS-L on several CNS cells including brain endothelial cells. Blocking integrins and chemokine gradients might be an interesting therapeutic target to prevent  $T_{\text{FH}}$  cells to be recruited to the site of tissue infection. We studied the expression of chemokine receptors and integrins among hypothalamic  $T_{\text{FH}}$  cells and found them to express CD49d, CCR5, and CD11a, but low levels of CCR7. Yet we did not perform experiments to investigate which of the integrins and chemokine markers are important for their transmigration to the hypothalamus.

In our model, the initial contact of effector CD8 T cells with orexin neurons resulted in tissue damage and the release of antigens in cervical lymph nodes. We observed that on day 12 after the transfer of autoreactive CD8 T cells in Orex-HA, but not WT mice, naïve HA-specific CD4 T cells were activated and proliferated in an antigen-specific manner in the cervical lymph nodes. These data suggest that the tissue damage results in the drainage of antigens to lymph nodes, where CD4 T cells can be activated and then migrate to the site of tissue damage. Nevertheless, in the scope of this thesis we could not prove that hypothalamic CD4 T cells were reacting against an antigen originating from orexinergic neurons, although about 19.5% of hypothalamic CD4 T cells from Orex-HA mice expressed the proliferation marker Ki-67. This might be an indication of local reactivation by an antigen, but further experiments are needed to clarify the nature of the recognized antigen(s).

In the overall picture, based on our observations we believe that CD4 T cell help is important for CD8 T cell i) differentiation ii) effective tissue invasiveness, and iii) persistence in the tissue. The figure below are intended to illustrate this, bearing in mind that the scenarios are partially hypotheses which we need to clarify in further experiments (**Fig 2.2**).



**Figure 3.2** CD4 T cells provide helper signals for the effective priming of CD8 T cells in the periphery during Pandemrix vaccination and their persistence at memory points in time in the hypothalamus

3.3 IFN $\gamma$  signaling is involved in neuronal destruction and CD8  $T_{RM}$  cell persistence in the CNS and other non-lymphoid tissues

We could show that IFN $\gamma$  deficiency in CD8 T cells impairs orexin neuron destruction. Several studies showed the importance of IFN $\gamma$  signaling for the killing of target cells. One study demonstrated that IFN $\gamma$ R-deficient CD8 T cells showed impaired lysis of target cells in a chromium release assay although they had normal stores of Perforin and Granzyme B (Tau et al. 2001). The authors discussed that IFN $\gamma$ R-deficiency in CD8 T cells leads to reduced  $Ca^{2+}$  influx, which subsequently leads to a reduced degranulation by CD8 T cells. Nevertheless, this hypothesis was never experimentally assessed according to my knowledge. Another team highlighted that autocrine IFN $\gamma$  signaling in CD8 T cells impairs the killing of keratinocytes both *in vivo* and *in vitro* by reducing their motility in the tissue (Bhat et al. 2017). In light of this, it would be of interest to study the localization of IFN $\gamma$ -deficient CD8 T cells in the hypothalamus. In the experimental design we would label IFN $\gamma^{+/+}$  CD8 T cells with a GFP- and IFN $\gamma^{-/-}$  CD8 T cells by a mCherry-expressing vector and perform immunofluorescence staining. On day 8 and day 30 we could study their localization to orexin neurons to see if IFN $\gamma^{-/-}$  CD8 T cells cannot locate close to their cellular target.

Besides this, IFN $\gamma$  is important for enhanced MHC-I expression in the target cells. Under normal conditions, most neurons do not express detectable levels of MHC-I molecules. Upon IFN $\gamma$ , but not TNF, stimulation the expression of MHC-I is induced on brain neurons, resulting in enhanced antigen presentation (Neumann et al. 1997). Similarly, IFN $\gamma$  released from autoreactive CD8 T cells in our model could have triggered MHC-I depending on the presentation of HA and subsequent cell lysis of orexin-producing neurons. We also confirmed the induced expression of *H-2K* on orexin neurons by translational profiling. H-2K<sup>D</sup> presents the immunogenic HA<sub>512-520</sub> peptide to CD8 T cells. Moreover, orexin neurons were stained by immunofluorescence for intranuclear phosphorylated pSTAT1 and showed surface expression of  $\beta$ 2-microglobulin as evidence of MHC-I expression.

Besides neurons, IFN $\gamma$  targets several other cell types in the brain. IFN $\gamma$  also induces the expression of MHC-II molecules and the expression of pro-inflammatory cytokines by microglia. The chronic intracerebroventricular administration of IFN $\gamma$  in mice resulted in depressive-like behavior and cognitive impairment accompanied by hippocampal neurodegeneration – hallmarks of AD and PD. Interestingly, once mice were treated with ruxolitinib, which inhibits the JAK/STAT1 pathway, microglial activation and neurogenic injury were reduced, and the cognitive impairment was alleviated (Zhang et al. 2020). In a rat model of PD induced by viral overexpression of  $\alpha$ -SYN, the application of the JAK inhibitor AZD1480 inhibited the loss of dopaminergic neurons. The drug reduced the activation of microglia and T-cell infiltration/persistence in the CNS (Qin et al. 2016).

This makes the inhibition of JAK inhibitors an interesting therapeutic target for several autoimmune diseases of the brain. It is worth noting that several JAK inhibitors can cross the BBB and could be tested as therapeutics for PD (Nabavi et al. 2019). Furthermore, the treatment of mice with the JAK inhibitor Berbamin during EAE resulted in the reduced encephalitogenic potential of CD4 T cells (Ren et al. 2008). Unfortunately, despite the existence of clinically approved STAT3 inhibitors, specific STAT1 inhibitors are yet to be developed (Hu et al. 2021, Chou et al. 2022). It would be interesting to test whether the treatment of Orex-HA mice with JAK or STAT1 inhibitors can reduce orexin neuron loss by impacting the number of CD8 T cells in the hypothalamus. This might present a treatment option for several CNS autoimmune diseases. Nevertheless, caution must be applied for the treatment of AD patients, as IFN $\gamma$  is important to recruit monocytes/macrophages, which reduce Amyloid- $\beta$  plaque deposition in the brain (Baruch et al. 2016).

Based on the analysis of the translational profiling of orexin neurons on day 8 (where the number of orexin neurons start to decrease) we identified a pathway upregulated in response to IFN $\gamma$  that was induced in an antigen-specific manner. Furthermore, HA-specific IFN $\gamma$ <sup>-/-</sup> CD8 T cells could not elicit neuronal loss in the vaccine model. In line with those results, the adoptive transfer of effector HA-specific IFN $\gamma$ <sup>-/-</sup> CD8 T cells also had a strongly impaired capacity to induce neuronal death upon their adoptive transfer.

Because IFN $\gamma$ <sup>-/-</sup> CD8 T cells were able to enter the hypothalamus as their IFN $\gamma$ -sufficient counterparts, we hypothesized that the reduced neuronal death could be due to impaired CNS recruitment of phagocytes. One study showed in an infectious setting, where CNS neurons were targeted by CD8 T cells that IFN $\gamma$ -mediated STAT1 activation in neurons results in the secretion of Ccl2 and Cxcl10, which drove the attraction of myeloid cells to virus-infected neurons (Di Liberto et al. 2018). Those recruited myeloid cells then phagocytosed synapses, resulting in neuronal loss. The authors found similar pSTAT1 staining in neurons of RE patients. Nevertheless, the depletion of CCR2<sup>+</sup> monocytes/macrophages during the disease course induced by IFN $\gamma$ -sufficient CD8 T cells could not prevent neuronal loss. Based on this experiment we cannot exclude the possibility that microglia are instead recruited and undergo synaptic stripping. For this, we bought PLX5622, a drug used to deplete microglia (Badimon et al. 2020). With this, we can test if other innate immune cells are involved in neuronal loss. We were also able to study the loss of orexin neurons in STAT1<sup>-/-</sup> Orex-HA mice.

Furthermore, our analysis of scRNA sequencing on day 30 in the adoptive transfer model revealed a cluster of CD8 T cells with IFN-signaling in the hypothalamus. Surprisingly, we observed that IFN $\gamma$ <sup>-/-</sup> CD8 T cells were not able to persist in the hypothalamus until day 30 and showed decreased expression of the tissue residency markers (CD69 and CD103). The co-transfer of IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ <sup>+/+</sup> CD8 T cells could not permit the persistence of IFN $\gamma$ <sup>-/-</sup> CD8 T cells in the CNS, suggesting a role for constant IFN $\gamma$  signaling for brain CD8 T<sub>RM</sub> maintenance. Indeed, histopathological data (intranuclear pSTAT1 staining) point towards constant IFN $\gamma$  signaling in hypothalamic CD8 T cells. Once IFN $\gamma$  signaling was abrogated or impaired by knock-out and knock-down of IFN $\gamma$ R1, respectively, the persistence of CD8 T cells in the hypothalamus was strongly reduced. IFN $\gamma$  signaling appeared to be important for the expression of several tissue-resident markers which are important for adhesion in the tissue, inhibiting tissue egress, and chemokine recognition.

Several studies aimed to identify which cytokines are important for the differentiation and maintenance of tissue-resident T cells. Regarding the skin, it was shown that naïve T cells that

were exposed to the biologically active form of TGF $\beta$  preferentially differentiated into skin epithelial T<sub>RM</sub> cells (Mani et al. 2019). Constant TGF $\beta$  signaling plays an important role not only for the naïve to effector transition but also for the effector to memory transition of CD8 T cells (Mackay et al. 2013). TGF $\beta$  drives the expression of CD103 on skin T<sub>RM</sub> cells and downregulates several tissue egress markers (Mackay et al. 2015, Christo et al. 2021). A recent study highlighted the importance of autocrine TGF $\beta$  signaling for T<sub>RM</sub> maintenance in the skin, impacting the persistence and expression of tissue residency markers CD69 and CD103. Interestingly, the competition for TGF $\beta$  accessibility of formerly formed antigen-specific T<sub>RM</sub> cells hindered bystander T<sub>RM</sub> cells from establishing in the skin (Hirai et al. 2021). In line with this, constant TGF $\beta$  signaling is also important for the persistence of CD8 T<sub>RM</sub> cells in the gut and the salivary gland, but not for CD8 T<sub>RM</sub> cells in the kidney, liver, and adipose tissues (Crowl et al. 2022). Concerning the liver, IL-15 signaling seemed to be essential for CD8 T<sub>RM</sub> maintenance (Holz et al. 2018), although it is likely that IL-15 signaling is important for the persistence of T<sub>RM</sub> cells in many non-lymphoid tissues as it was shown for the skin, the lung and gut (Reis et al. 2014, Mackay et al. 2015).

Our data suggest that autocrine IFN $\gamma$  signaling is important for CD8 T<sub>RM</sub> maintenance in the CNS. Furthermore, it might be that in the brain, as was shown for TGF $\beta$  in the skin, constant access to IFN $\gamma$  is crucial for antigen-specific CD8 T<sub>RM</sub> cells to compete against other CD8 T cells potentially filling this niche. This might explain why IFN $\gamma$ <sup>-/-</sup> CD8 T cells in the co-transfer experiment – possibly having limited access to constant IFN $\gamma$  signaling – lose the competition for the niche in the hypothalamus against their IFN $\gamma$ -sufficient counterparts. Creating a link, one might even hypothesize that IFN $\gamma$  signaling upregulates the expression of TGF $\beta$ R or IL15R, therefore making them more competitive in CNS persistence. This or another mechanism we want to further study is by sequencing IFN $\gamma$ R1-sufficient and deficient CD8 T cells in the CNS on memory points in time using the CRISPR-Cas9 experimental setting.

Regarding the CNS, the expression of IFN $\gamma$ -induced genes is elevated in several viral diseases and autoimmune diseases as mentioned earlier. This might support T<sub>RM</sub> persistence in the brain. One study highlighted that IFN $\gamma$  is crucial for the persistence of CD8 T<sub>RM</sub> cells in the brain of Sindbis virus-infected mice (Baxter et al. 2020). The proportion of CD8 and CD4 T cells expressing IFN $\gamma$  increased with time, reaching 80-90% on day 90 post-infection. Although IFN $\gamma$ - and IFN $\gamma$ R1-deficient CD8 T cells entered the brain in similar numbers as CD8 T cells from control mice, they showed deficiency in the expression of CD69 and CD103 on day 28 post-infection. Furthermore, during ZIKA or WNV infection, the study of Garber et al.



highlighted that CD8 T cells are the main source of IFN $\gamma$  in the brain, and loss of IFN $\gamma$  signaling reduces dramatically the loss of neurons in the hippocampus and improves learning defects (Garber et al. 2019). Nevertheless, given the effect of IFN $\gamma$  on many immune cells of the CNS, it is difficult to conclude from these studies, if this was due to CD8 T cell-intrinsic IFN $\gamma$  signaling. The global knock out of IFN $\gamma$ R1 has effect on every cell type of the body. Therefore, the lack of persistence of CD8 T cells in the brain cannot be attributed to intrinsic IFN $\gamma$ -signaling. Therefore, the adoptive transfer of IFN $\gamma$ R1-deficient CD8 T cells allowed us to study the role of an autocrine IFN $\gamma$  signaling loop for the persistence of CD8 T<sub>RM</sub> cells in the CNS.

Besides infectious diseases, CD8 T cells in the human brain respond to polyclonal stimulation to more than 90% by the production of IFN $\gamma$  (Smolders et al. 2018). Interestingly, in aged mice, CD8 T cells are found in proximity to oligodendrocytes and microglia that are stained positive for pSTAT-1. Single cell RNA sequencing revealed among oligodendrocytes several IFN-responsive genes and the injection of IFN $\gamma$  in aged mice resulted in a dramatic loss of oligodendrocytes and myelin in the brain (Kaya et al. 2022). In line with this, another study highlighted the accumulation of CD8 T cells in the CNS of aged mice, which express high levels of IFN $\gamma$  and show themselves IFN responsive gene signatures and pSTAT-1 staining (Martini et al. 2021).

In the context of neurodegenerative diseases of the CNS, A $\beta$ -specific T cells in AD patients are mostly IFN $\gamma$  producers (Pellicanò et al. 2010), and A $\beta$ -specific CD8 T cells in the brain of an AD mouse model produce high levels of IFN $\gamma$  (Browne et al. 2013). Furthermore, the CSF of PD patients shows upregulation of the type I cytokines and chemokines (Schröder et al. 2018) such as IFN $\gamma$ , CCL2 and CCL5 (Qin et al. 2016). High levels of IFN $\gamma$  are detected in chronic Parkinsonian macaques, and IFN $\gamma$  was important for disease progression in a mouse model for PD (Garcia et al. 2011). A recent study identified more than 60% of CD8 T cells in the brain parenchyma of PD stained positive for IFN $\gamma$  (Galiano-Landeira et al. 2020). Furthermore, elevated IFN $\gamma$ , CCL5, CXCL-10, and CXCL-9 levels were found in the brain of RE patients (Wang et al. 2022) and IFN $\gamma$ , as well as CXCL-9 levels positively, correlate with the time of disease onset (Owens et al. 2013). In the CSF of MS patients, expanded CD4 and CD8 T cell clones have enriched IFN signaling signatures compared to their counterparts in blood. Interestingly, T cells in the brain parenchyma of MS patients also show high expression levels of IFN $\gamma$  transcripts (Pappalardo et al. 2020). In sum, both in viral and brain autoimmune diseases, high levels of IFN $\gamma$  response genes are detected in the CNS.

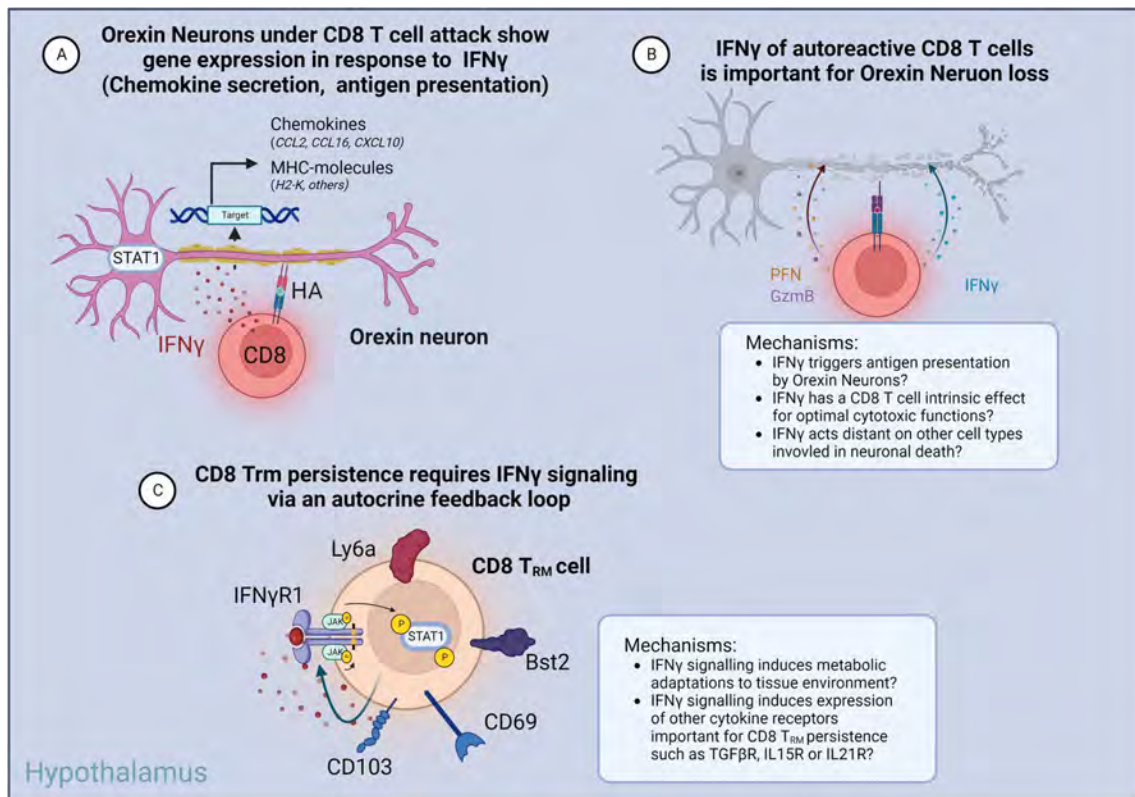
We demonstrated that impaired IFN $\gamma$  signaling impedes CD8 T<sub>RM</sub> persistence in the brain. We will now use the mouse model of *Toxoplasma gondii*-infected mice, which elicits a pronounced CD8 T cell response in the CNS, to assess whether the observed phenotype in an autoimmune setting can be reproduced in infectious settings.

The dependency of CD8 T<sub>RM</sub> persistence in non-lymphoid tissues on IFN $\gamma$  signaling might even be a more general observation, as we showed the decrease of CD8 T<sub>RM</sub> cells in the liver and the gut in a setting without infection or adoptive transfer of CD8 T cells. It is worthy of note that the study of Crowl et al. reported in the liver a large cluster of CD8 T<sub>RM</sub> cells with IFN signaling, similar to the gene expression profile we identified by scRNA sequencing of hypothalamic CD8 T cells (Crowl et al. 2022). A single-cell sequencing analysis in the gut during viral infection revealed a cluster with high expression of *IFNG*, *IFNGR1*, *NFKB*, and *BHLHE40* on D90 (Kurd et al. 2020). This study might even let us consider that IFN $\gamma$  signaling induces gene expression associated with metabolic adaptation to the tissue.

Besides, a recent study highlighted that reduced IFN signaling reduced the expression of CD103 and retention of CD8 T<sub>RM</sub> cells in the lung. This resulted in higher susceptibility to lung infections (Varese et al. 2022). In line with this, a study about the composition of T cells in the lung of elderly people revealed that aged people have reduced gene expression of type I interferons and IFN $\gamma$ . This correlates with a reduced number of CD8 T<sub>RM</sub> cells in the lung and increased susceptibility to lung infection with age, as discussed by the authors (Nguyen et al. 2021). Interestingly, T<sub>RM</sub> cells in the skin of psoriasis expressed highly the homing receptor CXCR3 sensing CXCL9 and CXCL10 (highly expressed by hair follicles), which might suggest that T<sub>RM</sub> cells trafficking in the tissue are dependent on IFN $\gamma$  induced chemokine gradients (Boniface et al. 2018). Furthermore, the skin of cutaneous lupus erythematosus (CLE) cases contains a high proportion of CD69 expressing CD4 and CD8 T cells, and IFN-signaling increased the level of CD69 expression of CD4 and CD8 T cells from PBMCs of CLE patients (Gu et al. 2021). In insulinitis lesions of the pancreas from type 1 diabetes (T1D) patients, an enhanced frequency of CD8<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> T<sub>RM</sub> cells has been observed. Within the pancreas of T1D patients, type I response genes including *IFNG*, *CXCL9*, *CCL5*, and *CXCL10* are up-regulated as compared to the pancreas from sex and age-matched controls (Kuric et al. 2017). Therefore, IFN $\gamma$  signaling might be important for the survival of CD8 T<sub>RM</sub> cells across various tissues.

Targeting IFN $\gamma$ R1 with pharmaceuticals could be an interesting target for chronic autoimmune diseases, for instance, progressive forms of MS where drugs targeting CNS entry (i.e. Natalizumab) are ineffective because the disease course is possibly driven by pathogenic T<sub>RM</sub> cells behind the BBB. For this, an optimal drug would deliver an IFN $\gamma$ R1 blocking antibody linked with a CD69 targeting single-chain nanobody across the BBB, to silence IFN $\gamma$  signaling in T<sub>RM</sub> cells. This might be safer than treating patients with IFN $\gamma$  blocking antibodies because IFN $\gamma$  is important for viral control of latent viruses of the CNS as described in 1.2.1. The blockade of IFN $\gamma$  would increase the risk of virus-induced encephalitis. Additionally, this blockade may expose patients to a high risk of infections outside the CNS during the treatment.

Furthermore, the combination of IFN $\gamma$ R1 blockade with Fingolimod or Natalizumab treatment might be important to prevent novel CD8 T cells from the periphery from entering the CNS and replenishing depleted T<sub>RM</sub> cells. A therapeutic strategy could be tested in our model to evaluate whether the niche is indeed repopulated by CD8 T cells coming from the periphery. By the depletion of CD4 T cells before the transfer of CD8 T cells, we could deplete CD8 T<sub>RM</sub> cells only when we simultaneously depleted CD8 T cells in the system. The number of CD8 T<sub>RM</sub> cells was not altered by CD4 T cell depletion, but their phenotype resembled less differentiated CD8 T<sub>RM</sub> cells. Bearing in mind these results, targeting both recirculating and tissue-resident T cells should be considered for any treatment that wants to target CD8 T cells in the CNS. The graphic below summarizes the above-mentioned findings (**Fig. 3.3**).



**Figure 3.3 IFN $\gamma$  signaling is involved in neuronal destruction and CD8 T<sub>RM</sub> cell persistence in the hypothalamus**

Taken together, the results of the Ph.D. Thesis shed light on how the interaction of CD4 and CD8 T cells induced hypothalamic infiltration and neuronal destruction. The chronic damage to orexin neurons was maintained by the presence of hypothalamic CD8 T<sub>RM</sub> cells. The depletion of those resulted in a partial restoration of neuronal loss. It appeared that IFN $\gamma$  signaling is crucial for the long-term persistence of CD8 T cells in the hypothalamus. From this, we would like to test JAK1/2 or STAT1 inhibitors in our T cell-mediated CNS autoimmune model. If those treatments are effective, these inhibitors might pave the way for future development of disease-modifying treatments for several brain autoimmune diseases.

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